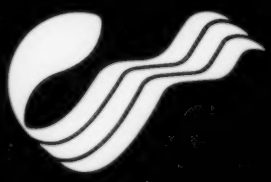

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Typeset and
Printed in Canada by: The Runge Press Limited, Ottawa

Composé et
Imprimé au Canada par: The Runge Press Limited, Ottawa

Design: Ludvic Saleh & Associates, Ottawa

Conception graphique: Ludvic Saleh & Associates, Ottawa

Publié sans interruption depuis 1901, le *Journal canadien des sciences halieutiques et aquatiques* a paru sous plusieurs titres : *Contributions to Canadian Biology* 1901–1925, *Contributions to Canadian Biology and Fisheries* 1926–1934, *Journal of the Biological Board of Canada* 1934–1937 et *Journal de l'office des recherches sur les pêcheries du Canada* 1938–1979.

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Canadian Journal of Fisheries and Aquatic Sciences

Journal canadien des sciences halieutiques et aquatiques

Volume 48, No. 9, September 1991 | **Volume 48, nº 9, septembre 1991**

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The *Journal* is abstracted or indexed in:/Le *Journal* est résumé ou signalé dans :

Aquatic Sciences and Fisheries Abstracts, Biological Abstracts, Chemical Abstracts, Current Awareness in Biological Sciences, Current Contents, FAO Freshwater and Aquaculture Contents Tables, FAO Marine Science Contents Tables, Oceanic Abstracts, and Science Citation Index

Isolation of a Y-Chromosomal DNA Probe Capable of Determining Genetic Sex in Chinook Salmon (*Oncorhynchus tshawytscha*)

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Devlin, R. H., B. K. McNeil, T. D. D. Groves, and E. M. Donaldson. 1991. Isolation of a Y-chromosomal DNA probe capable of determining genetic sex in chinook salmon (*Oncorhynchus tshawytscha*). Can. J. Fish. Aquat. Sci. 48: 1606–1612.

We have used subtractive hybridization methodologies to selectively clone a DNA fragment from the Y chromosome of chinook salmon (*Oncorhynchus tshawytscha*). This clone specifically detects a single 8-kb *Bam* HI restriction fragment in Southern blots of male genomic DNA that is absent in females. This sex-specific pattern was observed in over 50 individuals from five separate chinook stocks, suggesting that the organization of this sequence is conserved in this species. The strength of the hybridization signal, and the pattern observed in multiple enzyme digests, indicates that this sequence is repeated in the male chinook genome. With long autoradiographic exposures, fainter additional sequences can also be detected both in males and females, suggesting that other similar sequences not organized in a sex-specific fashion are found on the X chromosome and/or autosomes. Examination of the segregation of this DNA sequence in families showed that the male-specific pattern segregates from father to son, providing strong evidence that the DNA probe is from the Y chromosome. The application of Y-chromosomal probes to the commercial culture of this species will simplify the synthesis of new monosex strains and allow verification of existing genotypic female, phenotypic male stocks.

Nous avons utilisé des techniques d'hybridation par soustraction pour cloner sélectivement un fragment d'ADN du chromosome Y du saumon quinnat (*Oncorhynchus tshawytscha*). Ce clone s'hybride spécifiquement à un fragment de restriction unique *Bam* HI de 8 kb sur des Southern blots d'ADN génomique présent chez les mâles mais absent chez les femelles. Ce schéma lié au sexe a été observé chez plus de cinquante individus de cinq stocks distincts de saumon quinnat, ce qui semble indiquer que l'organisation de cette séquence est conservée chez cette espèce. La force du signal d'hybridation et le schéma observé lors de digestions multienzymatiques indiquent que cette séquence est répétée dans le génome du quinnat mâle. Une exposition prolongée des autoradiogrammes permet de détecter la présence de séquences supplémentaires correspondant à des signaux plus faibles chez le mâle et chez la femelle, ce qui semble indiquer que d'autres séquences similaires, dont l'organisation n'est pas liée au sexe, existent sur le chromosome X et/ou sur les autosomes. L'examen de la ségrégation de cette séquence d'ADN dans des familles permet de conclure à l'existence d'une ségrégation de père en fils, ce qui prouve que l'ADN de la sonde provient du chromosome Y. L'utilisation de sondes provenant du chromosome Y dans la culture commerciale de cette espèce de poisson simplifiera la création de nouvelles souches unisexuées et permettra l'identification des stocks existants de poissons de génotype femelle et de phénotype mâle.

Received March 18, 1991
Accepted May 17, 1991
(JA949)

Reçu le 18 mars 1991
Accepté le 17 mai 1991

Sex determination and differentiation have long been studied in a variety of fish species and have been attributed to both genetic and environmental factors (see Yamamoto 1969 and Bull 1983 for reviews). Among genetic systems, both multiple and single gene mechanisms have been described, and with the latter, sex determination may be associated with discrete sex chromosomes. Although morphologically distinct sex chromosomes (for example an X and Y or a Z and W chromosome pair) have been described cytogenetically in several fish species, in general, little morphological differentiation is observed (Gold 1979). This suggests that many teleosts exhibit a relatively primitive condition of sex-chromosome evolution where the accumulation of gross chromosomal rearrangements is insufficient to be visible at the cytological level. In salmonids, cytogenetically distinct chromosomes have been described for only a few species (Thorgaard 1977, 1978, 1983; Ueda and Ojima 1984a, and 1984b; Phillips and Ihssen 1985; Chourrout and Happe 1986; Shelenkova 1987; Lloyd and Thorgaard 1988;

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Frolov 1989), although genetic evidence has revealed that an XY system operates to control sex determination (Johnstone et al. 1979; Hunter et al. 1982; Chevassus et al. 1988).

In many vertebrate and invertebrate species, subtle molecular alterations occur to chromosome structure that result in significant differences in DNA sequence organization between sex chromosomes. These differences can take the form of simple mutations such as single-base-pair substitutions, insertions, or deletions, or they can be more substantial in the form of repetitive sequence amplifications of satellite DNAs or transposable elements. Indeed, sex-chromosome-specific DNAs have been isolated from a variety of species. The degree to which such molecular differences have played a role in the evolution of sex chromosomes in fish is unknown.

Salmonids possess a remarkably flexible system of sex differentiation that can be influenced by treatment with exogenous sex steroids. This lability to androgens or estrogens has facilitated the reproductive manipulation of these species for aquacultural purposes (Donaldson 1986). For example, in several salmonids, a proportion of males can precociously mature 1 yr before the bulk of the population, resulting in significant losses to the farmer due to an unmarketable or low-value product. For chinook salmon (*Oncorhynchus tshawytscha*) and rainbow trout (*Oncorhynchus mykiss*), this problem has been overcome by the production of all-female strains. Such monosex populations have been produced by sex-reversing normal (XX) females by androgen treatment during early development to generate phenotypic males that produce only X-bearing sperm (Hunter et al. 1983). This monosex milt is used to fertilize normal ova resulting in all-female offspring for use in production facilities, and a small number of XX zygotes can be masculinized to maintain a broodstock population of monosex males. This monosex milt technology can also be combined with triploidy induction to produce monosex female triploid salmonids which are reproductively sterile. The implementation and maintenance of monosex populations requires that genetic and phenotypic sex be independently discernible such that Y-chromosome-bearing normal males can be distinguished from phenotypic males which carry the female genotype (XX).

We have undertaken to isolate DNA probes from the salmon Y chromosome that are capable of distinguishing genetic males and females. Such probes will provide tools for the production and analysis of monosex fish and will allow a molecular genetic characterization of sex chromosome structure and function in these species. We have used the phenol enhanced reassociation technique (PERT) subtractive hybridization method (Kohne et al. 1977) to compete out the non-sex-specific sequences from male genomic DNA (with a large amount of female DNA) leaving an enriched fraction of male-specific sequences available for cloning. Kunkel et al. (1985) have used the PERT method to isolate human X-chromosomal sequences from the muscular dystrophy locus, and a similar technique has been used previously to successfully isolate Y-chromosomal sequences from the mouse (Lamar and Palmer 1984). In this report, we describe the isolation of a DNA probe that can easily identify Y-chromosome-bearing individuals in chinook salmon. We show that this sequence is repetitive in nature, that it is found in all chinook stocks tested, and that it segregates faithfully from father to son in family analyses.

Materials and Methods

Chinook salmon samples were obtained from the following five sources within British Columbia. (1) Big Qualicum River on Vancouver Island. Five mature adults of each sex were obtained on November 4, 1989, liver samples were taken and stored frozen at −70°C, and gametes were collected from each individual. A 5 × 5 mating scheme was performed where all individuals of one sex were mated to all individuals of the other sex. At 9 mo of age, F_1 progeny from each family group were dissected to identify either ovary or testis, and a liver sample was retained from each individual. (2) Chilliwack River, a coastal tributary of the Fraser River. Thirty individuals from the 1989 brood were sexed by dissection and liver samples were taken. (3) Quinsam River on Vancouver Island. Two mature individuals were collected on November 19, 1990, and liver samples were retained. (4) Nicola River, an interior tributary of the Thompson River. Two individuals were sexed on December 21, 1990, and liver samples were retained. (5) One albino fish of each sex was sampled from an aquaculture stock of uncertain origin.

In general, molecular biological methods were performed as described by Sambrook et al. (1989). DNA was prepared by digesting approximately 100 mg of chopped liver tissue in 5 mL of proteinase K buffer (10 mM Tris, 10 mM ethylenediaminetetraacetic acid (EDTA), 1% sodium dodecyl sulphate (SDS), 200 μg proteinase K/mL, pH 8.0) at 37°C overnight. After salt precipitation of remaining protein by adding NaCl to 1.5 M, the sample was centrifuged at 10 000 × *g* for 10 min. The supernatant was extracted once with phenol – chloroform – isoamyl alcohol (50:50:1) and precipitated with 2.5 volumes of ethanol. The precipitate was transferred to a 70% ethanol wash, collected by centrifugation, dried briefly under vacuum, and dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

Subtractive hybridization was performed as described by Kunkel et al. (1985) utilizing one male and one female chinook salmon from the Big Qualicum stock. A large excess of randomly sheared DNA (250 μg, reduced to a size of between 500 and 8000 base pairs (bp)) from a single female was employed to remove the non-sex-specific sequences from a small amount of male DNA (1 μg) that had been digested with *Mbo* I. The hybridization reaction was conducted in 2.5 mL and contained boiled male and female DNAs, 1.25 M $NaClO_4$, 120 mM sodium phosphate, pH 6.8, and 12% phenol (the phenol was equilibrated to pH 7.5 with Tris base). The reaction was shaken on a Vortex Genie (Fisher) at setting 2.5 for 6 d at room temperature. After annealing, the hybridization mixture was extracted once with chloroform – isoamyl alcohol (50:1). The aqueous phase was precipitated twice and then dialyzed against TE buffer and precipitated with 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol. The precipitate was washed in 70% ethanol, dried, and redissolved in TE buffer. This process yielded a population of double-stranded molecules that could be recovered by ligation into the phagemid vector Bluescript II (Stratagene) that had been cut with the restriction enzyme *Bam* HI and dephosphorylated with bacterial alkyline phosphatase. Individual colonies were picked and minipreparations of their plasmid DNAs were screened for the presence of inserts by restriction analysis. Clones containing suitably sized inserts were redigested and these fragments were isolated from low-melting agarose gels. Each of these fragments was hybridized separately to Southern blots containing three male and three female genomic DNAs that had been digested either with *Bam* HI or with *Hae* III.

For Southern blot analysis, 5 μg of genomic DNA was digested with the appropriate restriction enzyme in 30 μL at 37°C overnight and then subjected to electrophoresis in 0.5%

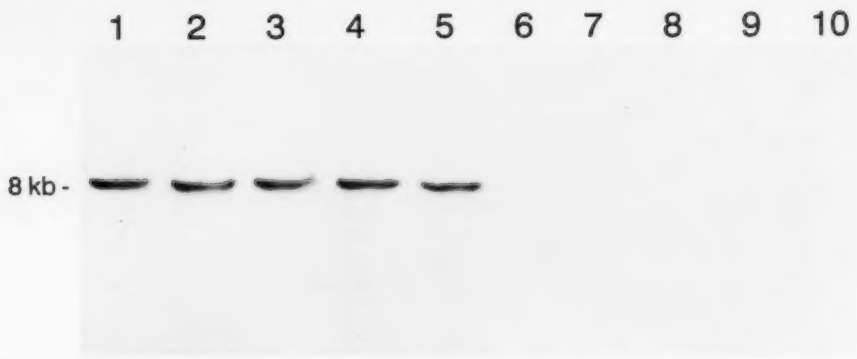

FIG. 1. Southern blots of male (lanes 1–5) and female (lanes 6–10) chinook DNA digested with *Bam* HI and probed with the OtY1 male-specific DNA fragment.

TABLE 1. Summary of chinook stocks molecularly sexed with the OtY1 probe.

Stock	Location	Phenotypic Sex	OtY1 positive	OtY1 negative
Big Qualicum	Vancouver Island	Male	11	0
		Female	0	11
Chilliwack	Fraser Valley	Male	15	0
		Female	0	15
Quinsam	Vancouver Island	Male	1	0
		Female	0	1
Nicola	Interior B.C.	Male	2	0
		Female	0	1
Albino	Uncertain	Male	1	0
		Female	0	1
Total		Male	30	0
		Female	0	29

agarose gels. After partial hydrolysis in 0.25 M HCl for 15 min, the gels were denatured in 1.5 M NaCl, 0.5 M NaOH for 1 h and transferred to Hybond-N membrane (Amersham) as described by Meinkoth and Wahl (1984). Membranes were prehybridized for at least 1 h in Church and Gilbert (1984) buffer at 55°C and then hybridized overnight under the same conditions to radioactive probes (derived from PERT fragments isolated from low-melting agarose gels) labelled with ^{32}P by the method of Feinberg and Vogelstein (1983). Filters were washed twice in 2 × SSC (150 mM sodium chloride, 15 mM sodium citrate, pH 7.0), 0.1% SDS, twice at room temperature, twice at 55°C, and then exposed to X-ray film (Kodak XAR) with a single intensifying screen at −70°C for 3–48 h.

Results

PERT subtractive hybridization procedures were used to selectively enrich for male-specific sequences in chinook salmon. Of over 250 putative PERT clones analyzed, 18 had inserts of a size useful for analysis. When hybridized to Southern blots containing male and female chinook genomic DNA, most of the probes showed either a single-copy pattern with no variation or a repetitive pattern with individual variation lacking any sex specificity. However, one of the probes (a 250-bp fragment) revealed a striking pattern of male specificity, detecting a 8-kb *Bam* HI restriction fragment in males but not in females. To verify this observation, *Bam* HI digests of genomic DNA were analyzed from five animals of each sex from the Chilliwack River stock, and a clearly sex-specific pattern was again observed (Fig. 1). To examine whether this pattern was stock specific in chinook salmon, we analyzed DNA from an additional 20 individuals (equal numbers of males and females) from the Chilliwack River, five males and five females from the Big Qualicum stock, a male and a female from the Quinsam River, two individuals from the Nicola River (an interior form of chinook showing genetic differentiation from the coastal forms; R. Withler, J. Blackburn, and C. Clarke, Pacific Biological Station, Hammond Bay Road, Nanaimo, B.C. V9R 5K6, pers. comm.), and two individuals from an albino stock of uncertain origin. All of these fish showed a molecular phenotype consistent with their gonadal sex determined from dissection (Table 1). We have named this probe OtY1.

To study the organization of this sequence further, Southern blots of male and female DNA that had been digested with a variety of other restriction enzymes were hybridized with OtY1. Many enzymes that recognize a 6-bp sequence produced discrete bands in a similarly male-specific pattern (Fig. 2). After longer autoradiographic exposure (on dot blots, not shown), it was clear that homologous sequences could in fact be detected at low levels in females as well. Thus, although sequences with OtY1 homology do not appear to be limited to males, the organization of them into intense restriction fragments appears to be a male-specific characteristic in chinook salmon. The strength of the autoradiographic signal indicated that the OtY1 sequence was repetitive in nature in males, since clear signals could be detected in as litle as 3 h. The repetitive nature of this probe is also revealed by the presence of multiple bands occurring on Southern blots with restriction enzymes that do not cut within the OtY1 sequence.

The above results established that the repeat pattern of OtY1 is associated with males in wild populations. To formally demonstrate linkage to the Y chromosome, the segregation of this marker was examined in a family of Big Qualicum chinook. Figure 3 shows a Southern analysis of female and male parents and of five sons and five daughters. The results clearly show the segregation of the OtY1 marker from father to son, and because little mortality occurred in this family that could result in differential survival of male and female offspring, the result also demonstrates the linkage of this repeat pattern to the Y chromosome.

Some commercial populations of monosex chinook salmon are currently producing male progeny in significant numbers.

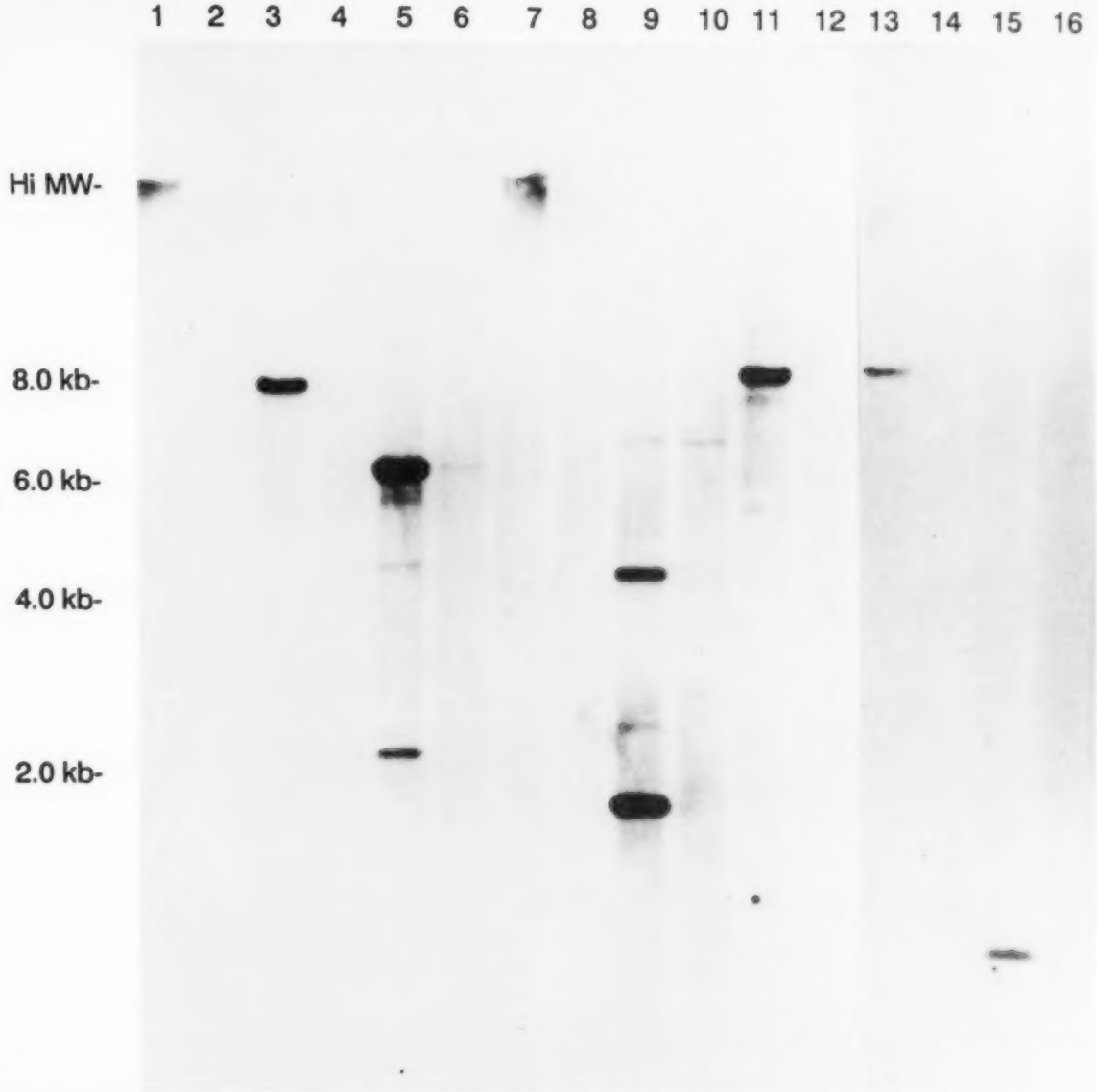

FIG. 2. Southern blots of male (odd-numbered lanes) and female (even-numbered lanes) chinook DNA digested with a variety of restriction enzymes and probed with OtY1. Lanes 1 and 2, *Apa* I; lanes 3 and 4; *Bam* HI; lanes 5 and 6, *Eco* RI; lanes 7 and 8, *Eco* RV; lanes 9 and 10, *Hin* cII; lanes 11 and 12, *Hin* dIII; lanes 13 and 14, *Kpn* I; lanes 15 and 16, *Pst* I.

Broodstock individuals were obtained from a monosex population that was producing some exceptional males to determine whether they were of the XX chromosomal constitution typical of monosex stocks or if they were in fact regular Y-bearing males that had been accidentally introduced into the monosex broodstock populations. Of four such individuals examined with the OtY1 probe, one contained a Y chromosome (Fig. 4). We therefore conclude that at least some males arise in monosex populations as a result of contamination with regular XY males from other sources. However, a more thorough investigation of male exceptions will be required to eliminate the possibility that genetic change in XX individuals (i.e. accumulation of male-determining modifiers) is responsible in some cases.

Discussion

We have described the isolation of a DNA probe from the Y chromosome of chinook salmon and confirmed that molecular genetic differences do exist between the sexes in salmonids. Examination of this marker in chinook families revealed an exclusive segregation from father to son, strongly suggesting linkage to the Y chromosome. Further, investigation of over 50 individuals from several natural populations has failed to identify any exceptions to this rule. Because cytological evidence in salmonids is consistent with the X and Y chromosomes sharing extensive regions of homology, and because genetic evidence has shown that at least one sex-chromosome-linked

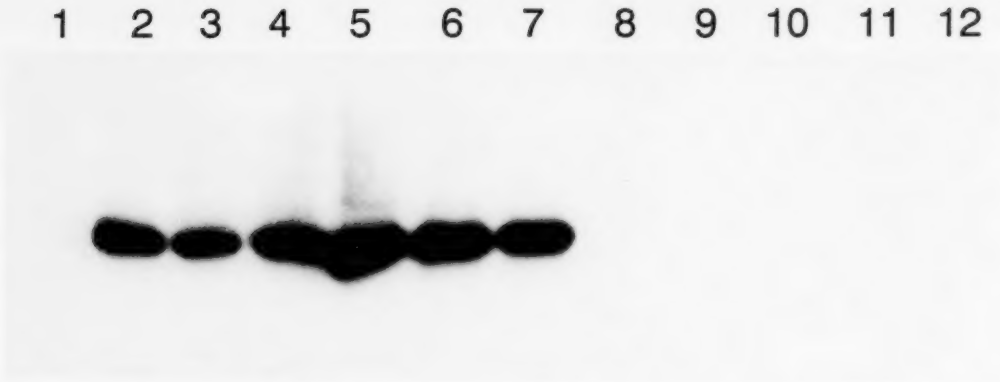

FIG. 3. Analysis of OtY1 segregation in a chinook salmon family. A Southern blot was prepared with *Bam* HI digested DNA from female (lane 1) and male (lane 2) parents and from five male (lanes 3–7) and five female (lanes 8–12) offspring of known phenotypic sex.

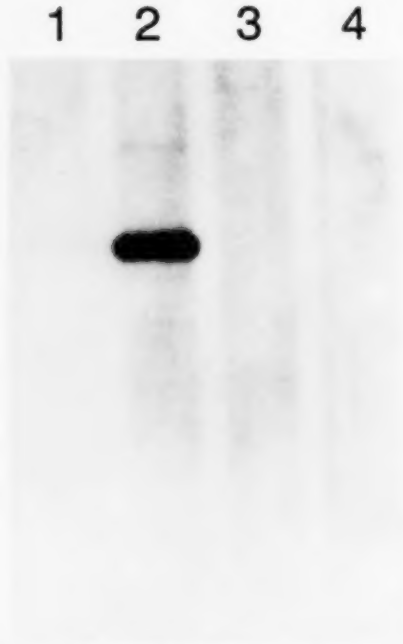

FIG. 4. Southern blot of *Bam* HI digested DNA from four putative genotypic female, phenotypic male chinook salmon broodstock probed with OtY1.

locus, *hexosaminidase*, is closely linked but still capable of undergoing genetic exchange with the sex-determining locus (Gellman et al. 1987), our failure to detect any recombinant genotypes suggests that the OtY1 probe is probably physically located close to the sex-determining genes. If the OtY1 sequence is organized at a single genetic locus on the Y chromosome, and regular genetic exchange can occur between the sex chromosomes, this genetic distance (no recombinants in 50 individuals examined) could represent a physical distance of 2 Mbp of DNA or less based on information derived from other vertebrates (Botstein et al. 1980). However, it is also possible that this sequence is dispersed, or is distantly located from the sex-determining genes on the Y chromosome. In this case, strong linkage to the sex-determining genes may arise from suppressed crossing over due to chromosomal rearrangements or centromere-proximal map positions. Evidence has been presented which suggests that chromosome fusion and/or rearrangement has occurred during sex chromosome evolution in salmonids (May et al. 1989).

The evolution of heteromorphic sex chromosomes is often accompanied by a variety of DNA alterations that are probably not involved in the sex determination process. Lucchesi (1978) and Charlesworth (1978) reviewed possible modes of sex chromosome evolution, describing the progression from an ancestral condition where both the X and Y (or Z and W) chromosomes are isosequential except at the sex-determining locus to a condition where these chromosomes have differentiated to a degree that they become cytologically distinct. This process may occur through a genetic mechanism called "Muller's ratchet" (Charlesworth 1978), where random mutations and rearrangements on the sex-specific chromosome cumulatively become fixed in the population. As a consequence, these chromosomes may comprise large amounts of degenerate DNA that is either chromosome specific or organized in a fashion not found on other chromosomes. This degeneration process will be most pronounced immediately adjacent to the sex-determining locus because mutations induced further away have an increased chance of crossing over onto the homologous chromosome and being eliminated from the population in homozygous form. Degenerate DNAs are often repetitive in nature, either as simple satellite sequences, complex repeats, or transposable elements, and may be associated with genetically inert chromosomal regions, termed heterochromatin (Brutlag 1980; Devlin et al. 1990a, 1990b). It is interesting that Phillips et al. (1985) have utilized Q banding to detect heterochromatic regions within chinook chromosomes, and a polymorphic one of these may be associated with sex chromosomes. It is possible that the repetitive sequence that we have isolated is derived from this chromosomal region.

Sex-chromosomal DNAs have been isolated from a variety of other species from several other phyla and are composed of both single-copy and repetitive sequences. For example, a variety of repetitive sex-specific or sex-associated DNAs have been described in *Drosophila* (Goldstein et al. 1982; Lifschytz and Hareven 1982; Steinemann 1982; Livak 1984; Waring and Pollack 1987; Pardue et al. 1987). In particular, the sequences described by Livak (1984) are organized in a similar fashion to

those described in the present study: homologous sequences are present in both sexes, but the organization of those on the Y chromosome is distinct from those found elsewhere in the genome. Remarkably, another sequence, Bkm, detects may repetitive and highly variable repeats and hybridizes to the sex chromosomes of both invertebrates and vertebrates (Singh et al. 1984). Nanda et al. (1990) have used a portion of this sequence to identify sex-specific squences in some strains of guppies (*Poecilia reticulata*) and have used in situ hybridization methods to localize them to a single Y chromosome. In humans, repetitive sex chromosomal sequences have also been isolated (Cook 1976; Kunkel et al. 1976; Cook et al. 1982; Wolfe et al. 1984; Muller et al. 1986), and single-copy sequences (ZFY and SRY) with putative sex-determining function have been described (Page et al. 1987; Sinclair et al. 1990). Similar studies have revealed Y-chromosome-specific sequences in mice (Lamar and Palmer 1984; Mitchell et al. 1989; Gubbay et al. 1990). Some of these sequences have been tested for their presence in salmonids (Ferreiro et al. 1989; Lloyd et al. 1989), and although single-copy and repetitive signals were detected, no evidence for sex-specific organization was observed. We have also examined both ZFY and Bkm for sex-specific organization in chinook salmon and have found no consistent differences (unpublished observations).

Manipulation of sex and reproduction has become an important tool in aquacultural and fisheries biology. For example, all-female populations eliminate the problems associated with early maturation of males and can be produced either by direct feminization (Hunter et al. 1986) or by indirect methods that utilize genetics to control sex. Genetically monosex female populations of salmon are synthesized either by gynogenesis (Refstie et al. 1982) or by crossing masculinized genotypic females (androgen treated) with regular females (Hunter et al. 1983). In the latter case, masculinized females (phenotypic males) must be distinguished from regular (XY) sibling males. Although phenotypic sex in submature salmonids can be determined by morphometric analysis (see Beacham and Murray 1986), determination of genetic sex has usually been accomplished by a test cross to determine the sex of resulting offspring. For many aquaculture operations, this process is excessively expensive and time consuming to perform.

The existence of a DNA probe from the Y chromosome of chinook salmon will greatly facilitate monosex production by allowing rapid detection of phenotypic males that are lacking the male-specific DNA and are genetically XX females. Although monosex technology represents a major advance in salmonid husbandry, occasional stocks of monosex fish produce low levels of males within their populations. This occurrence of males has presumably arisen either through the accumulation of male modifiers in an otherwise XX female background or by accidental substitution of regular (XY) males in place of monosex (XX) males. We have shown that, at least in some cases, the appearance of males in monosex female production populations has occurred by the accidental introduction of regular males into the parental broodstock populations. Thus, this problem can now be rectified by routine screening of monosex broodstock populations in aquaculture facilities with the Y-chromosome probe to ensure that no genotypic male fish are utilized. To further facilitate application of this probe to the aquaculture industry, we have recently sequenced the OtY1 probe and have developed a rapid polymerase chain reaction assay that can determine genetic sex from small amounts of blood or fin tissue in a single day. Such analyses, coupled with molecular genetic studies of the chinook Y chromosome, will benefit the aquaculture industry and improve our understanding of the molecular basis of sex determination in fish.

Acknowledgements

We thank Inge van Oostveen for excellent laboratory assistance, Igor Solar, Ruth Withler, and Ewen McLean for reading the manuscript, and the reviewers for their helpful comments. We acknowledge financial assistance for this work from the Science Council of British Columbia and from the Department of Fisheries and Oceans.

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A System-of-Equations Approach to Modeling Age-Structured Fish Populations: The Case of Alaskan Red King Crab, *Paralithodes camtschaticus*

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This paper presents a simultaneous system-of-equations approach to modeling age-structured populations using trawl survey age/size frequency data. The analysis builds upon a Ricker spawner–recruit structure and provides a cohort-based estimation method that retains the underlying dynamic properties of a delay-difference model. The framework shares a common spawner–recruit function across age-class equations. This exploits the commonality among cohort members and serves as an instrumental variable, lessening the effect of measurement errors in estimation. The dynamic features of the underlying age-structured population are retained through age-specific net survivability and growth parameters that link age-classes. The technique uses multiple observations on a cohort to further mitigate the effect of measurement error and improve overall estimation efficiency. A seemingly unrelated regression estimation method is required to address contemporaneous correlation of errors across age-classes. This framework is applied to trawl survey data for adult male Alaskan king crab, *Paralithodes camtschaticus*.

Les auteurs présentent un système d'équations simultanées pour modéliser des populations structurées selon l'âge à l'aide de données sur la fréquence des âges selon l'âge à l'aide de données sur la fréquence des âges selon la longueur recueillies au cours de relevés par chalutage. L'analyse repose sur une structure géniteurs–recrues élaborée par Ricker et constitue une méthode d'évaluation basée sur les cohorts qui retient les propriétés dynamiques sous-jacentes d'un modèle de délai des différences. La structure fondamentale partage une fonction commune géniteur–recrue dans les équations relatives aux classes d'âge. Ceci tire parti du facteur commun entre les membres des cohortes et sert de variable contribuante qui réduit l'incidence des erreurs de quantification dans les estimations. Les caractéristiques dynamiques de la population sous-jacente structurée selon l'âge sont retenues par l'entremise du taux de survie net spécifique à l'âge et les paramètres de croissance qui lient les classes d'âge. La méthode utilise des observations multiples d'une cohorte pour réduire encore plus les erreurs de quantification et améliorer l'efficacité globale des estimations. Une méthode d'estimation de la régression, apparemment sans rapport, est nécessaire pour étudier la corrélation contemporaine des erreurs parmi les classes d'âge. Cette structure fondamentale est appliquée à des données de relevés par chalutage relatives à des mâles adultes du crabe royal rouge (*Paralithodes camtschaticus*).

Received June 26, 1990
Accepted March 13, 1991
(JA628)

Reçu le 26 juin 1990
Accepté le 13 mars 1991

An extensive literature on age/size-structured fish population models has developed in recent years in part because age and/or size frequency catch data are readily available. Modern age/size analytical methods are rooted in virtual population analysis (Gulland 1965; Murphy 1965; Pope 1972; Ricker 1975). As the limitations of virtual population analysis became apparent, it evolved into variants of catch-at-age analysis (Doubleday 1976; Paloheimo 1980; Fournier and Archibald 1982; Collie and Sissenwine 1983; Deriso et al. 1985, 1989). More recently, size-structured frameworks have been proposed for situations where either catch-at-age data do not exist, but size frequency data do exist, or where the accuracy of size-at-age estimates comes into question (Schnute 1987; Schnute et al. 1989a, 1989b; Sullivan et al. 1990; Parma and Deriso 1990).

Modeling age-structured Alaskan red king crab, *Paralithodes camtschaticus*, stocks in the Bristol Bay portion of the Bering Sea unfortunately cannot benefit much from this rich literature. Commercial catch statistics do not report the distribution of catch by size or age. Rather gross harvest weight and numbers are reported in this males-only fishery. Also, grading by size occurs during secondary processing outside of Alaska (primarily in Japan and Seattle). The National Marine Fisheries Service (NMFS), however, conducts annual trawl surveys to estimate stock abundance. These trawl survey data are reported both in terms of a size frequency distribution and an age frequency distribution that is calculated from a length–age key. Male and female stocks are reported separately.

There are two obvious approaches to modeling age-class abundance from the trawl survey data. First, a delay-difference structure like that proposed by Deriso (1980) could be used. A recruitment function would be estimated for, say, 5-yr-olds, and advanced to subsequent age-classes with age-specific mortality and growth parameters. This approach has the advantage of yielding an exact representation of age-structure effects on biomass dynamics, but relies on the accuracy of age-specific

estimates of stock. Alternatively, one could estimate a series of separate and independent age-class recruitment functions. Parameter estimates would differ across age-class equations reflecting altered recruitment trajectories due to cumulative growth and mortality. This procedure has the distinct advantage of basing the estimates of individual age-class abundance on estimates of aggregate parental stocks, but does not explicitly model the underlying age-specific growth and mortality parameters as age-class advances.

Both approaches are theoretically sound and should, in the absence of measurement errors, yield virtually identical predictions. But fisheries data tend to be measured with error, and this certainly is the case with the king crab trawl survey data. The NMFS data suffer from two predominant types of error: survey error and errors related to converting carapace size to estimates of crab age. Errors in the crab data are obvious, since there are a number of cases where the population of a cohort (in numbers) increases as it advances in age. For example, the reported population of male 5-yr-olds in 1978 was 22.0 million, while the 1979 population of male 6-yr-olds was 20% greater, 26.5 million.

Walters and Ludwig (1981) and Ludwig and Walters (1981) initially addressed this problem of errors in variables when estimating stock–recruitment relationships. They approached the problem by incorporating independent assessments of environmental and measurement error variances. Ludwig and Walters (1989) extended their earlier analysis, comparing the generalized Ricker (1954) family of recruitment functions with those of Deriso (1980) and Schnute (1985). They concluded that in the presence of large data errors, the trade-off between bias and variance requires one to "give up fidelity of the estimation model to the underlying dynamics in order to improve estimation performance" (p. 142). The Ricker class of models was found to outperform the Deriso–Schnute family of models.

This paper takes an alternative approach — one that exploits beneficial attributes of both Ricker- and Deriso-type models. To do so, however, requires addressing not only measurement errors, but also requires adopting a cohort-based estimation scheme. The measurement error problem is addressed with an instrumental variables approach similar to that of Ludwig and Walters (1989). That is, individual right-hand-side age-class variables are replaced by more aggregated (less errant) "instruments" that are correlated with the variable of interest, but measured with less error. A Ricker-type spawner–recruit model is used for this purpose. However, unlike Ludwig and Walters (1989), individual age-class equations are not estimated independently. Rather, the model developed in this paper views each age-class within a system of simultaneous equations that share a common spawner–recruit function. Each age-class equation is linked through age-specific net survivability and growth parameters, retaining the dynamic features of the underlying age-structured population.

The "systems" approach introduces contemporaneous correlation of errors across age-classes. Accounting for inter-age-class correlations that occur within each time step of a cohort requires the use of an estimation technique known as seemingly unrelated regression.

The framework developed here is applied to modeling age-structured, adult male red king crab stocks in the Bristol Bay portion of the Bering Sea. The analysis uses NMFS published size/age distribution data developed from the 1968–89 trawl surveys.[1] We limit the analysis to adult male red king crab for several reasons. First, king crab recruit into the parental stock at approximately age 5 whereas they recruit into the fishery at ages 8 or 9 and can live to 14+ yr of age. Second, this is a males-only fishery. Third, trawl survey data for juveniles are grossly inaccurate because of survey gear design.

Methods

Conceptual Framework

The conceptual basis for modeling age-class recruitment is illustrated in the system-of-equations (1) for 5- to 8-yr-old male crab:

$$
\begin{aligned}
& M_{5,t} = f(F_{t-6}, M_{t-6}; \beta) + \mu_{5,t} \\
(1)\quad & M_{6,t+1} = \tau_6 M_{5,t} + \mu_{6,t+1} \\
& M_{7,t+2} = \tau_7 M_{6,t+1} + \mu_{7,t+2} \\
& M_{8,t+3} = \tau_8 M_{7,t+2} + \mu_{8,t+3}.
\end{aligned}
$$

The variables in (1) defined as $M_{a,t}$, for ages $a = 5, 6, 7, 8$, are age-specific recruitment measured in biomass; $f(F_{t-s}, M_{t-s}; \beta)$ represents a spawner–recruit function; $F_{t-s} = \sum_{a=5}^{14} F_{a,t-s}$ and $M_{t-s} = \sum_{a=5}^{14} M_{a,t-s}$ represent parental stock biomass in year $t-s$, the year of spawning, where $F_{a,t-s}$ and $M_{a,t-s}$ represents the biomass of females and males of age-class a in year $t-s$; β represents the parameter vector of the spawner–recruit function; τ_6, τ_7, and τ_8 represent 5- to 6-, 6- to 7-, and 7- to 8-yr-old net survivability/growth rates, where $\tau_a = \exp(z_a)$ with z_a representing the survivability/growth parameter for age-class a; and $\mu_{5,t}, \ldots, \mu_{8,t+3}$ represent the respective process errors in the progression of the cohort.

The dynamic features of the underlying age-structured population are retained via a longitudinal view of a cohort. That is, the system emulates a delay-difference equation similar to that proposed by Deriso (1980); age-specific net survivability and growth parameters link the age-classes.

If the population values in (1) were observed without error, and assuming the classical process error properties were maintained, the usual least squares approach to estimating the parameters in (1) could be pursued. However, given that the population observations are subject to measurement error, the least squares approach applied to (1) would yield biased and inconsistent parameter estimates. In order to clarify the estimation problem involved, let

$$(2)\quad M^o_{a,t} = M_{a,t} + v_{a,t},\ a = 5, \ldots, 8$$

represent the deviation between an observed population level of crab ($M^o_{a,t}$) and the actual population level ($M_{a,t}$), where $v_{a,t}$ represents measurement error. Furthermore, assume for the moment that the observed level of aggregate parental stock is

[1]Age and size categories are used interchangeably throughout this paper. Following NMFS age/carapace length composition data, age-specific adult male crab (M_a) conform to the following size distribution: M_5 = 95–109 mm, M_6 = 110–119 mm, M_7 = 120–129 mm, M_8 = 130–139 mm, and $M_{9+} \geq 140$ mm.

accurate. Then, representing (1) in terms of observable variables yields (3):

$$
\begin{aligned}
M^o_{5,t} &= f(F^o_{t-6}, M^o_{t-6}; \beta) + (\mu_{5,t} + v_{5,t}) \\
M^o_{6,t+1} &= \tau_6 M^o_{5,t} + (\mu_{6,t+1} + v_{6,t+1} - \tau_6 v_{5,t}) \\
M^o_{7,t+2} &= \tau_7 M^o_{6,t+1} + (\mu_{7,t+2} + v_{7,t+2} - \tau_7 v_{6,t+1}) \\
M^o_{8,t+3} &= \tau_8 M^o_{7,t+2} + (\mu_{8,t+3} + v_{8,t+3} - \tau_8 v_{7,t+2}).
\end{aligned} \tag{3}
$$

It is clear from (2) and (3) that the explanatory variables are correlated with the (composite) disturbance terms of the estimable equations; unbiased and consistent least squares estimates of τ_6, τ_7, and τ_8 will not be forthcoming.

This problem can be mitigated by replacing the explanatory variables with closely correlated instrumental variables, so long as the instruments are not closely correlated with the error terms. A suitable candidate to replace $M^o_{a,t}$ is a common spawner–recruit function adjusted for cumulative growth and mortality, which allows (3) to be rewritten as (4):

$$
\begin{aligned}
M^o_{5,t} &= f(F^o_{t-6}, M^o_{t-6}; \beta) + (\mu_{5,t} + v_{5,t}) \\
M^o_{6,t+1} &= \tau_6 f(F^o_{t-6}, M^o_{t-6}; \beta) + (\mu_{6,t+1} + v_{6,t+1} + \tau_6 \mu_{5,t}) \\
M^o_{7,t+1} &= (\tau_7 \tau_6) f(F^o_{t-6}, M^o_{t-6}; \beta) \\
&\quad + (\mu_{7,t+2} + v_{7,t+2} + \tau_7 \mu_{6,t+1} + \tau_7 \tau_6 \mu_{5,t}) \\
M^o_{8,t+3} &= (\tau_8 \tau_7 \tau_6) f(F^o_{t-6}, M^o_{t-6}; \beta) \\
&\quad + (\mu_{8,t+3} + v_{8,t+3} + \tau_8 \mu_{7,t+2} + \tau_8 \tau_7 \mu_{6,t+1} + \tau_8 \tau_7 \tau_6 \mu_{5,t}).
\end{aligned} \tag{4}
$$

Equation (4) illustrates two facets of commonality among individual age-classes within a cohort that requires a "system-of-equations" perspective. First, male 5-yr-old crab in year t, male 6-yr-olds in year $t+1$, male 7-yr-olds in year $t+2$, and male 8-yr-olds in year $t+3$ are all spawned by the same parental stock. The practical implication of this commonality is that each member of the cohort shares a common spawner–recruit trajectory from year $t-6$ (the time period in which the cohort was spawned) to year t (the time period in which the cohort was 5 yr old). This is represented in (4) by the identical spawner–recruit function $f(F^o_{t-6}, M^o_{t-6}; \beta)$ shared among all age-class equations. Second, age-class advancement requires only an accumulation of age-specific survivability/growth effects. This is represented in (4) by the age-specific survivability/growth coefficients, τ_a, which are shared across equations and multiplied together as age-class advances to represent the cumulative effect of growth and mortality.

An alternative representation of the system in (4) that expresses the observations on dependent variables in identical time periods is shown in (5):

$$
\begin{aligned}
M^o_{5,t} &= f(F^o_{t-6}, M^o_{t-6}; \beta) + (\mu_{5,t} + v_{5,t}) \\
M^o_{6,t} &= \tau_6 f(F^o_{t-7}, M^o_{t-7}; \beta) + (\mu_{6,t} + v_{6,t} + \tau_6 \mu_{5,t-1}) \\
M^o_{7,t} &= (\tau_7 \tau_6) f(F^o_{t-8}, M^o_{t-8}; \beta) \\
&\quad + (\mu_{7,t} + v_{7,t} + \tau_7 \mu_{6,t-1} + \tau_7 \tau_6 \mu_{5,t-2}) \\
M^o_{8,t} &= (\tau_8 \tau_7 \tau_6) f(F^o_{t-9}, M^o_{t-9}; \beta) + (\mu_{8,t} + v_{8,t} \\
&\quad + \tau_8 \mu_{7,t-1} + \tau_8 \tau_7 \mu_{6,t-2} + \tau_8 \tau_7 \tau_6 \mu_{5,t-3}).
\end{aligned} \tag{5}
$$

Assuming the process and measurement errors have zero means and finite variances, nonlinear least squares yields consistent and asymptotically unbiased parameter estimates. (For the sake of simplifying notation, the superscript o, which indicates observed, is dropped from the remainder of the paper.)

This system-of-equations approach to age-class estimation raises the issue of estimation efficiency gains that can be achieved by accounting for the contemporaneous correlation of the systemwide error structure. Survey errors, size-at-age errors, and unmeasured environmental perturbations are likely to be systematically related across equations in any given time period. That is, disturbances across equations may exhibit some degree of common movement and, hence, correlation. Zellner (1962) first recognized the gains in efficiency that can be achieved by incorporating information regarding contemporaneous correlation of the error terms into the estimation process. He suggested applying generalized least squares jointly to the system-of-equations. This approach, known as Seemingly Unrelated Regression (SUR), simultaneously estimates all parameters in the system-of-equations, rather than equation by equation. Additional detail on the SUR process is described in the Appendix.

In cases where sample sizes are large, even higher gains in estimation efficiency might be obtained by incorporating information regarding autocorrelation among the error terms of the various age-class equations within a cohort (recall the composite error terms of (4)). However, for small sample sizes often found in the analyses of commercial fisheries, the use of these more complicated estimation procedures is not recommended. The more complicated error covariance structure cannot, in general, be accurately estimated, and the theoretical gains in estimation efficiency are not obtained in practice (Judge et al. 1985, p. 496–497).

Complete specification of adult male stocks requires estimation of 9- to 14-yr-old males. These six age-classes are modeled as a single, aggregate age-class, $M_{9\text{–}14,t}$. There are three principal reasons for adopting this aggregate age-class approach for male 9- to 14-yr-olds. First, the age-class is managed as a composite class of legal crab; management does not, nor is it expected to, discriminate among these larger size categories. Second, individual age/size-class models similar to $M_{5,t}$ to $M_{8,t}$ would exhaust too many degrees of freedom. The lagging structure of parental stock within a spawner–recruit function results in a loss of one additional observation for each successive age-class. Specification of a 14-yr-old equation would require a 15-yr lag between parental stock and recruits. Third, isolating the transition from 8- to 9-yr-olds would require a tenuous assumption concerning the age/size distribution of harvest, for which there are no data. Accordingly, $M_{9\text{–}14,t}$ is specified in (6) as a simple function of unharvested male 8- to 14-yr-old crab in the previous period ($M_{8\text{–}14,t-1}$ − Harvest$_{t-1}$), adjusted by a composite survivability/growth coefficient ($z_{9\text{–}14}$):

$$
M_{9\text{–}14,t} = (M_{8\text{–}14,t-1} - \text{Harvest}_{t-1}) \exp(z_{9\text{–}14}) + \mu_{9\text{–}14,t}. \tag{6}
$$

The $M_{9\text{–}14,t}$ equation (6) does not share parameters with other age-class equations. However, contemporaneous correlation of errors links this equation with the system specified in (4) or (5). Accordingly, the aggregate $M_{9\text{–}14,t}$ equation should be included in the SUR estimation process.

Conceptual Framework Benefits

In general, the systems approach to modeling age-structured fish populations offers a variety of conceptual advantages. For one, it retains the aggregation benefits of a spawner–recruit function. The shared spawner–recruit function anchors age-class population estimates to observations on the aggregate

parental stock, rather than to inherently less accurate observations on a single prerecruit age-class. Aggregation lessens the effect of size/age errors in explanatory variables. The accuracy of the aggregate observed parental stock of 5- to 14-yr-olds is not affected if, for example, a 7-yr-old crab is mistakenly classified as an 8- or 6-yr-old. Aggregation can also mitigate other randomly distributed errors in the measurement of the 10 individual age-class populations from which the aggregate 5- to 14-yr-old observation is derived.

A second benefit is that the systems approach incorporates observations across age-classes within a cohort to estimate age-class recruitment. For example, four observations per cohort would be simultaneously considered in (4) or (5). Use of several observations on a cohort lessens the influence of any single observation on the estimation of the spawner–recruit function, or on any of the survivability/growth parameters. In essence, the systems approach reduces the incentive for closely fitting an aberrant observation. It does so by considering the additional errors between the estimated and the observed populations of other members of that cohort.

The cohort framework offers other conceptual advantages. The number of observations available for estimation is greatly increased by pooling cross-sectional and time series data. This benefit is especially important to this research, since there are only 22 yr of trawl survey data. Pooling information across four age-classes effectively quadruples the number of observations. Moreover, the number of equation-specific parameters decreases relative to a single, independent equation approach in which separate spawner–recruit functions, each with its own parameters, are estimated for each age-class. Degrees of freedom are gained with the system-of-equations approach, which allows tighter confidence intervals to be constructed around parameter estimates.

Empirical Estimation

Empirical estimation of the system conceptualized in (5) and (6) hinges on specifying the common spawner–recruit function. A variety of alternative specifications were examined, ranging from the simple Ricker models in which parental stock is aggregated to specifications that attempt to differentiate between the contribution of male and female parental stocks. Decomposition of parental stocks by sex yielded a significantly better overall statistical fit. The common spawner–recruit specification given in (7) provided the best statistical fit:

$$(7)\quad M_{a,t} = \beta_0 F_{t-(a+1)}^{\beta_1}\exp(\beta_2 F_{t-(a+1)} + \beta_3 FM_{t-(a+1)} + \beta_4 M_{t-(a+1)})\exp(z_a) + \mu_{a,t}^{*}$$

where $a = 5, 6, 7,$ and 8; $\exp(z_a) = \prod_{j=5}^{a} \tau_j$, $\tau_5 = 1.0$; and $\mu_{a,t}^{*}$ represents the composite disturbance term. Male recruitment into the 5-yr-old age-class in period t is a function of composite adult female stock ($F_{t-(a+1)}$), adult male stock ($M_{t-(a+1)}$), and the product of adult females and adult males ($FM_{t-(a+1)}$), all lagged to the year of conception ($t-(a+1)$ years). All variables are measured in millions of pounds of biomass. β_0 through β_4 are parameters to be estimated, where β_1 is a flexibility parameter included to accommodate the possibility of increasing as well as decreasing average reproductive productivity.

Attempts to represent either the leading term or the exponential expression in terms of lagged aggregate parental stock ($F_{t-(a+1)} + M_{t-(a+1)}$) failed. This representation performed very poorly in estimating the level of recruits, which points to the importance of the female broodstock. Females can be bred only once a year, while a single male can breed many females. Nevertheless, contribution of adult males to reproductive success cannot be overlooked and is captured in the exponential term. This spawner–recruit functional specification suggests that breeding success, and thus recruitment, may be a function of not only the individual stock of adult males and females, but also the relative sex composition of adult crab.

The system implied by (6) and (7), or equivalently (5) and (6), was modified for empirical analysis in a fundamental way. Differential mortality was hypothesized for the period beginning in 1981. The Alaskan king crab fishery experienced a boom–bust phenomena reminiscent of many commercial fisheries. Statewide harvests more than tripled between 1968 and 1980, culminating in a record catch of 180 million pounds. The Bristol Bay harvest rose from 8.7 million pounds (1 pound = 0.454 kg) in 1968 to a peak of 130 million pounds in 1980. In 1983, the Bristol Bay fishery was closed — statewide harvests plummeted to 17 million pounds by 1985, and remain depressed today. Otto (1986) attributed the collapse, at least in part, to an increase in mortality. Accordingly, a structural break commencing in 1981 was investigated.

Mortality shifts were accommodated in the male 6-, 7-, 8-, and 9- to 14-yr-old equations by shifting the survivability/growth coefficients. In order to reflect a change in mortality affecting juvenile crab, all parameters of the Ricker function were allowed to change. Separate recruitment functions were specified for the period prior to 1981 and following 1980. A variety of less flexible mortality adjustments also were investigated, as well as different terminal periods of increased mortalities.

Results

The model ultimately used to estimate age-class recruitment is presented in (8):

$$M_{5,t} = (\beta_0 + \beta'_0 I_0) F_{t-6}^{\beta_1 + \beta' 1 I_0} \exp((\beta_2 + \beta'_2 I_0) F_{t-6} + (\beta_3 + \beta'_3 I_0) FM_{t-6} + (\beta_4 + \beta'_4 I_0) M_{t-6}) + \mu_{5t}^{*}$$

$$M_{6,t} = (\beta_0 + \beta'_0 I_1) F_{t-7}^{\beta_1 + \beta' 1 I_1} \exp((\beta_2 + \beta'_2 I_1) F_{t-7} + (\beta_3 + \beta'_3 I_1) FM_{t-7} + (\beta_4 + \beta'_4 I_1) M_{t-7}) \exp(z_6 + z'_6 I_0) + \mu_{6t}^{*}$$

$$(8)\quad M_{7,t} = (\beta_0 + \beta'_0 I_2) F_{t-8}^{\beta_1 + \beta' 1 I_2} \exp((\beta_2 + \beta'_2 I_2) F_{t-8} + (\beta_3 + \beta'_3 I_2) FM_{t-8} + (\beta_4 + \beta'_4 I_2) M_{t-8}) \exp(z_6 + z_6' I_1 + z_7 + z'_7 I_0) + \mu_{7t}^{*}$$

$$M_{8,t} = (\beta_0 + \beta'_0 I_3) F_{t-9}^{\beta_1 + \beta' 1 I_3} \exp((\beta_2 + \beta'_2 I_3) F_{t-9} + (\beta_3 + \beta'_3 I_3) FM_{t-9} + (\beta_4 + \beta'_4 I_3) M_{t-9}) \exp(z_6 + z'_6 I_2 + z_7 + z'_7 I_1 + z_8 + z'_8 I_0)) + \mu_{8t}^{*}$$

$$M_{9\text{–}14,t} = (M_{8\text{–}14,t-1} - \text{Harvest}_{t-1}) \exp(z_{9\text{–}14} + z'_{9\text{–}14} I_4) + \mu_{9\text{–}14t}^{*}$$

The parameters z'_6, z'_7, z'_8, and $z'_{9\text{–}14}$ were added to the existing growth/mortality parameters in the 6-, 7-, 8-, and 9- to 14-yr-old equations to accommodate the mortality break. All parameters in the spawner–recruit function were allowed to change by incorporating $\beta'_0, \ldots, \beta'_4$. Cross-equation restric-

tions in the form of identical parameters (β_0 through β_4 and β'_0 through β'_4) are induced by the spawner–recruit function being shared across age-classes; individual survivability/growth coefficients (z_a and z'_a) appearing in more than one equation also are restricted to being identical. The I_j are indicator variables equalling 1.0 if t = 1980 + j through 1989, for j = 0, 1, 2, and 3, otherwise I_j = 0.0. I_4 is an indicator variable equalling 1.0 if t = 1981 through 1985, otherwise I_4 = 0.0. The time subscript t = 1977, 1978,...,1989. The error structure is specified $E(\mu^*_{ht}, \mu^*_{kt}) = \sigma_{hk}$ for $h \neq k$ and h, k = 5, 6, 7, 8, and 9–14. All other variables are as previously defined. The SAS Institute Inc. (1984) nonlinear SUR procedure, SYSNLIN, was used for model estimation.

The first year of data used in model estimation is 1977 for two reasons. First, the SAS-SUR procedure requires that the entire system span a common time period. Second, the 9-yr lag defining parental stock of 8-yr-olds established 1977 as the first year for which a complete set of observations on all age-classes is available.

Three observations in the data series are treated as outliers: the 1977 observations on 5- and 6-yr-olds and the 1988 observation on 9- to 14-yr-olds. The 5- and 6-yr-old stock observations appeared suspicious because their values were nearly twice the adjacent 1976 and 1978 observations. Regarding the 9- to 14-yr-old observation, the number of 8- to 14-yr-olds in 1988, net of harvest, represents an upper bound to the abundance of 9- to 14-yr-olds the following year. However, the 1989 NMFS population estimate of 9- to 14-yr-olds exceeds this upper bound by 42%. Outlier tests confirmed the status of these observations as outliers at the 0.05 level of Type I error.[2] Rather than eliminating the 1977 and 1988 observations by marking them with indicator variables, cubic splines were fit to the respective age-class data with the outlying observations removed. Observed biomass values of 80.71, 71.82, and 29.05 were replaced in the data set with 50.25, 51.48, and 56.02 for male 5-, 6-, and 9- to 14-yr-olds, olds, respectively, as indicated by the respective cubic splines.

Model estimates are presented in Table 1 — the checks indicate age-classes that share the corresponding parameter estimates. The t-ratios indicate whether a particular parameter is significantly different from 0.0, except for β_0 and β_1 which were tested for a significant difference from 1.0.

All parameters, with the exception of β'_0, z_6, z'_7, and z_{9-14} are significant at the 0.02 level. The marginal Type I error probabilities at which the hypotheses β'_0, z_6, and z'_7 equal zero would be rejected are 0.35, 0.07, and 0.25, respectively. The parameters are, nonetheless, retained at their estimated values to prevent the possibility of committing a Type II error from omitting relevant variables, which would result in model bias. Interest in subsequent use of the model estimates for forecasting crab stocks suggests that model bias be avoided. The survivability/growth coefficient z_{9-14} is not statistically different from zero; a zero value indicates that growth exactly offsets mortality. The coefficient is retained at its estimated value rather than imposing the restriction that growth exactly offsets mortality.

All parameter signs conform to a priori expectations. Goodness-of-fit statistics indicate that the predictive accuracy of each age-class is quite good. R^2 increases as age-class advances, ranging from 0.76 for the 5-yr-old equation to 0.99 for the 9- to 14-yr-old equation. Theil inequality coefficients range from 0.25 for the 5-yr-old equation to 0.08 for the 9- to 14-yr-old equation, suggesting that the model predicts turning points quite well.[3] Note, also, that the system degrees of freedom are 47. A single, independent equation approach would yield from 3 to 11 degrees of freedom, and consequently, much wider confidence intervals.

TABLE 1. SUR dynamic recruitment model with shared parameters (indicated by √) for 5-, 6-, 7-, 8-, and 9- to 14-yr-old male Alaskan red king crab (millions of pounds, 1968–89).

Parameter	Estimate	t-ratio	M_5	M_6	M_7	M_8	M_{9-14}
β_0	0.036	42.96	√	√	√	√	
β'_0	1.607	0.94	√	√	√	√	
β_1	3.184	7.30	√	√	√	√	
β'_1	−2.309	−4.56	√	√	√	√	
β_2	−0.075	−9.61	√	√	√	√	
β'_2	0.053	4.09	√	√	√	√	
β_3	0.138	5.74	√	√	√	√	
β'_3	−0.102	−3.39	√	√	√	√	
β_4	−0.016	−7.02	√	√	√	√	
β'_4	0.014	4.78	√	√	√	√	
z_6	−0.125	−1.87		√	√	√	
z'_6	−0.274	−3.18		√	√	√	
z_7	0.193	2.83			√	√	
z'_7	−0.151	−1.16			√	√	
z_8	0.188	3.77				√	
z'_8	−0.429	−3.42				√	
z_{9-14}	0.010	0.50					√
z'_{9-14}	−0.707	−5.81					√
Goodness-of-fit statistics							
R^2			0.759	0.891	0.972	0.964	0.988
U			0.254	0.175	0.107	0.130	0.078

NOTE: t-statistics on β_0, and β_1 test the null hypothesis that the coefficient = 1.0. U is the Theil inequality coefficent (see text footnote 3 for details). Goodness-of-fit statistics were simulated over the estimated model. The 1977 observation of 5- and 6-yr-olds and the 1988 observation of 9- to 14-yr-olds were replaced with cubic spline estimates. System degrees of freedom = 47.

Discussion

Figures 1 and 2 further illustrate the excellent systemwide fit. The predictive accuracy of the systems approach is evident by comparing observed versus predicted estimates on the basis of crab numbers rather than biomass, at least for the single age-class models (Fig. 1). This avoids any confusion due to partially compensatory growth and mortality, as age-class advances. The aggregate 9- to 14-yr-old results (Fig. 2) are illustrated in biomass because of an inability to disaggregate this composite age-class.

Figure 1 brings attention to the principal strength of this type of cohort analysis. An independent equation approach imposes a single, and possibly misguided, standard of predictive accuracy — closeness of fit relative to only those individual observations on the particular age-class. Such a singular criterion is

[2]The observations in question were marked with indicator variables; t-tests indicated that the parameters were significantly different from 0.0 at the 0.05 level.

[3]The U-inequality ($0 \leq U \leq 1$) was developed by Theil (1961) to evaluate the turning point accuracy of simulation forecasts. A coefficient of zero is associated with a perfect forecast whereas a coefficient of 1.0 implies that the model is no better than a status quo forecast of zero change in the observed value. Pindyck and Rubinfeld (1981) provided a good discussion of the various Theil forecast error statistics.

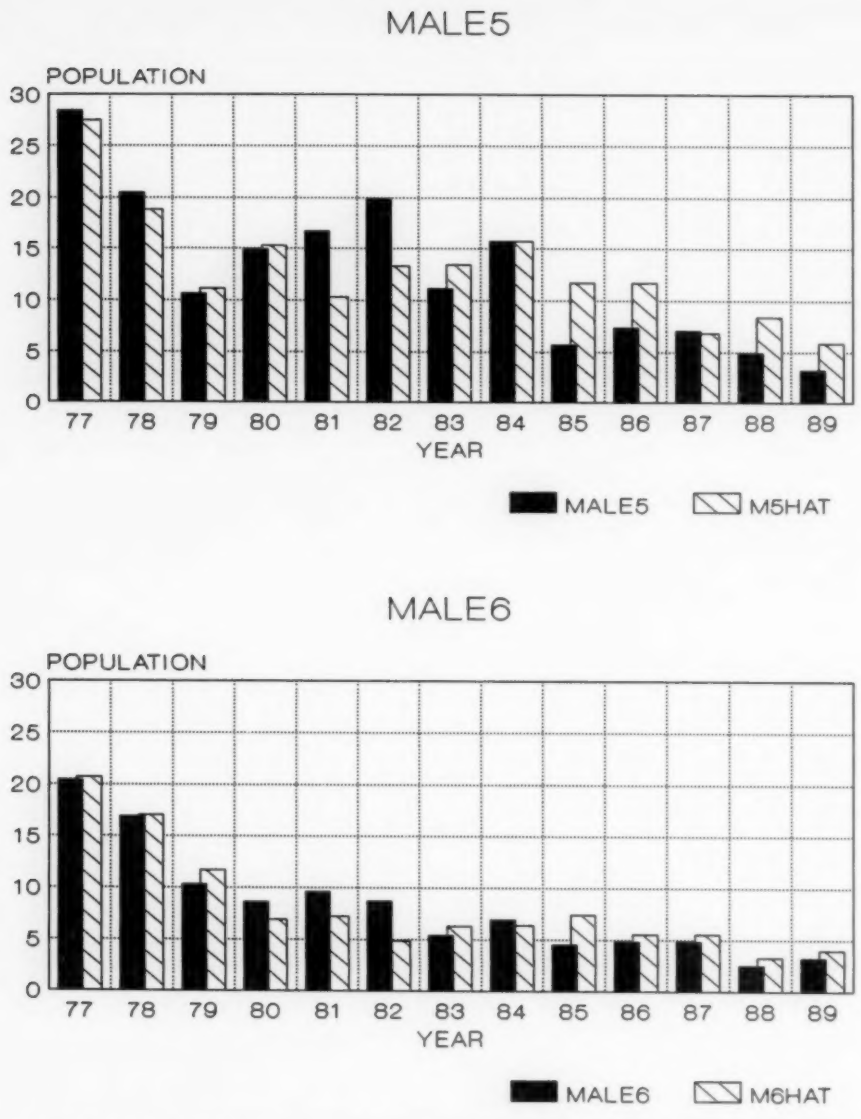

FIG. 1. SUR predicted versus observed population estimates (millions) of 5- to 8-yr-old males.
(*Fig. 1 concluded next page*)

inherently flawed when the underlying trawl survey data are known to contain errors.

The systems approach to stock estimation imposes a second criterion on parameter estimation and model performance. Closeness of fit relative to an individual observation within any age-class is tempered by the influence of that estimate on prior and/or subsequent age-classes. For example, consider Fig. 3. The estimate of the 1982 population of 5-yr-olds underestimates the observed population of 6.4 million crab. However, examination of the cohort reveals that the 1983–85 estimates of male 6-, 7-, and 8-yr-olds, respectively, are close to the observed populations. Comparing survey data observations suggests an extremely high, probably unrealistic, mortality rate of 73% affecting male 5-yr-olds in 1982. A strength of the cohort framework is that it smooths these types of extremes. Consistency across a cohort may necessitate substantial deviations between estimated and observed population for a single age-class.

The systems approach avoids the aforementioned problem of increasing cohort population over time provided that mortality rates are positive. Although mortality rates are not estimated directly, they are recoverable from the growth/survivability parameters z_a. NMFS estimates of weight by age, Wt_a, can be used to convert the z_a to an explicit mortality estimate, as shown in (9)[4]:

$$(9)\quad m_{a,t} = 1 - \exp(z_a + z'_a I_0)(\mathrm{Wt}_a/\mathrm{Wt}_{a+1}), \qquad a = 6, 7, 8$$

where $m_{a,t}$ is the mortality rate of age a crab in time t; all other variables are as previously defined. The term $(\mathrm{Wt}_a/\mathrm{Wt}_{a+1})$ can-

[4]NMFS estimates that male 5-, 6-, 7-, and 8-yr-old crab weigh 1.77, 2.52, 3.31, and 4.27 pounds (0.81, 1.14, 1.50, and 1.94 kg), respectively.

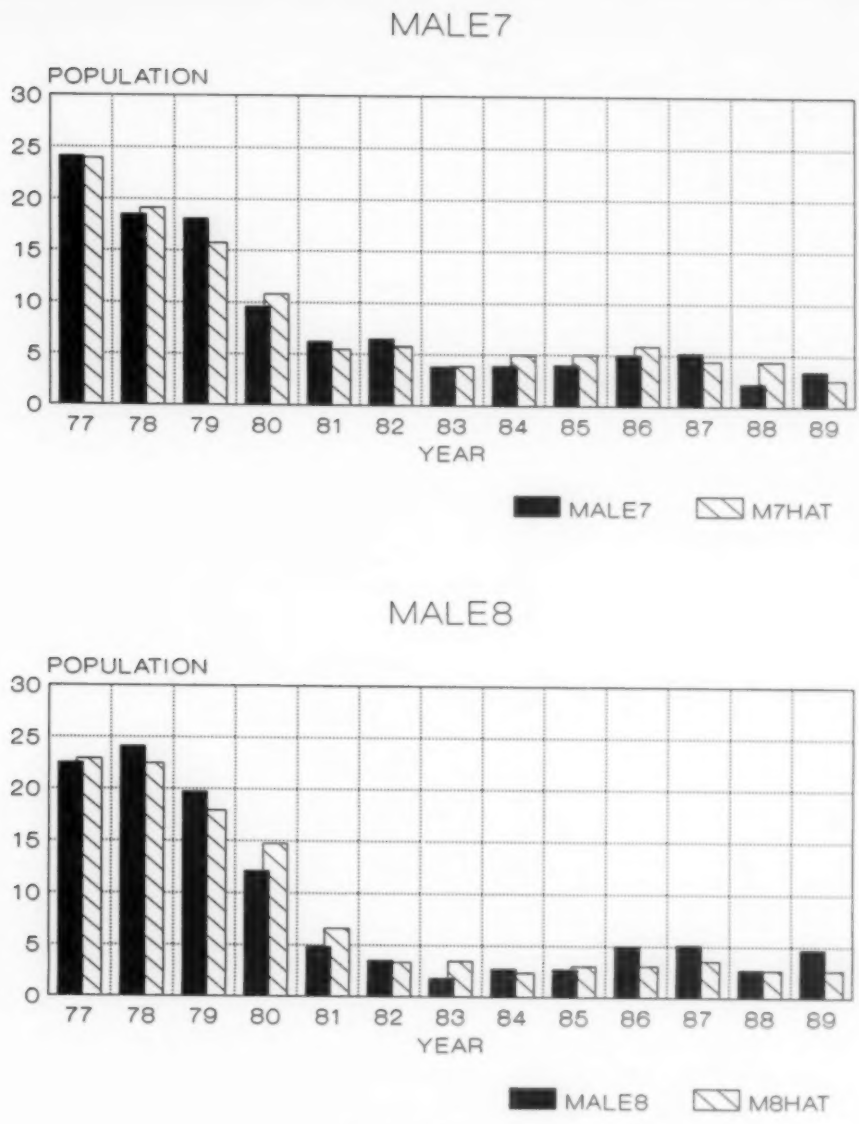

FIG. 1. *(Concluded)*

cels the implicit growth component in the growth/survivability rate.

Average annual natural mortality rates by age are provided in Table 2 for the time periods 1977–80 and 1981–89. Comparison of the rates for the two time periods reveals a pronounced structural shift in mortality beginning in 1981. The 5-yr-old mortality rate increases from 38.0 to 52.9%, the 6-yr-old rate increases from 7.6 to 20.6%, and the 7-yr-old rate increases from 6.5 to 39.1%.

While the aggregate survivability/growth coefficient for male 9- to 14-yr-olds cannot be converted to a pure mortality estimate, a considerable increase in mortality beginning in 1981 can be inferred. The survivability/growth rate for this age-class decreased from 101% (growth slightly exceeding mortality) to 49.9% (mortality twice the rate of growth).

An increase in mortality played a substantial role in the decline of the king crab stocks in the early 1980's. However, mortality for the aggregate 9- to 14-yr-old age-class appears to have returned to "normal" by 1985. In contrast, the results suggest that younger age-classes continue to suffer high mortality rates. Why a structural shift in mortality for the two groups would span different time periods is unclear. The results, however, are consistent with the observed trawl survey data and fishery performance, as reflected by harvest and weight per unit effort.

Conclusion

The framework presented here takes a systems approach to population estimation based on cohort analysis. The systems approach estimates a single, shared spawner–recruit function

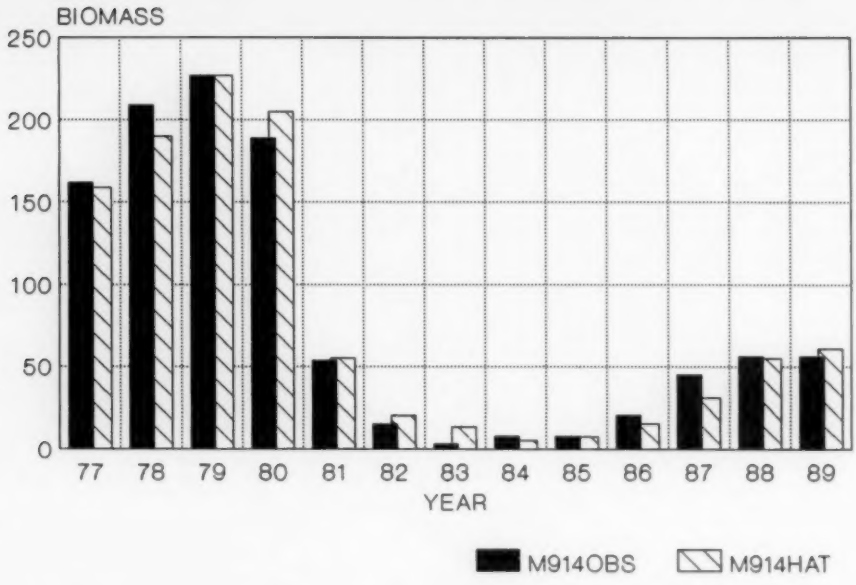

FIG. 2. SUR predicted versus observed biomass estimates (millions of pounds) of 9- to 14-yr-old males.

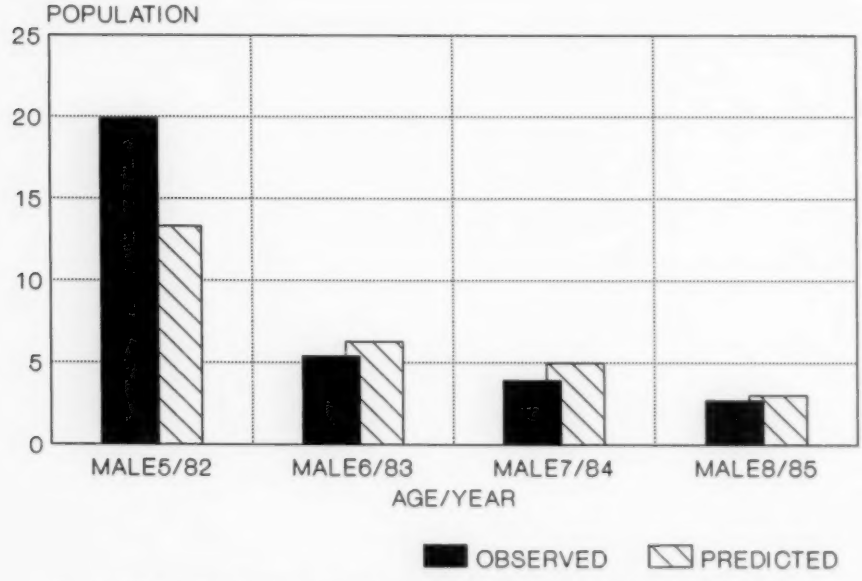

FIG. 3. SUR predicted versus observed population estimates (millions) of the 1982 cohort.

TABLE 2. Estimated age-specific male annual mortality rates by time period.

Age (yr)	Mortality rate 1977–80	1981–89
5–6	0.380	0.529
6–7	0.076	0.206
7–8	0.065	0.391

which explicitly recognizes that all members of a cohort derive from a common parental stock. Thus, all observations on a cohort are used simultaneously in estimating the spawner–recruit function. This approach contrasts with the traditional Ricker framework in which individual age-class populations are modeled as unique, independent recruitment functions.

The systems approach offers a variety of operational features which mitigate problems endemic to population modeling of fisheries, e.g. brief data series, inconsistent or errant survey observations on size/age-structured populations, and difficulties in measuring mortalities. The brief data series available for

the Bristol Bay king crab fishery, 1968–89, coupled with lengthy time lags required by the spawner–recruit framework, provide few observations for model estimation. However, the systems approach simultaneously utilizes all information on a cohort by pooling cross-sectional and time series data, thereby greatly increasing the number of effective observations. This permits more detailed, parameter-intensive specifications while mitigating the usual associated degrees of freedom problems, and it can lead to gains in parameter estimation efficiency.

The systems approach advances the findings of Ludwig and Walters (1989) by retaining fidelity with the underlying biomass dynamics while addressing the errors in variables problem endemic to much fisheries data. It does so by blending features of a spawner–recruit model with that of a delay-difference model. The spawner–recruit component subsequently serves as an instrumental variable which is estimated based on several successive observations of a cohort, as opposed to a single observation per year. This feature is particularly advantageous in that the NMFS trawl survey data contain a variety of inconsistencies due to a combination of survey error and problems inherent in using carapace size to estimate crab age. Instances of extremely high, low, and even negative mortalities (cohort population increasing over time) are present in the data series. By pooling all observations on a cohort, the influence of any single observation on the estimated spawner–recruit function is reduced. This implicit smoothing feature acts to produce biologically consistent age-class estimates which could not be assured with either an independent equation approach to spawner–recruit function estimation or with a delay-difference approach. The SUR estimation method also allows one to address the problem of contemporaneous correlation among age-class errors.

Acknowledgements

This research was funded in part by the National Marine Fisheries Service, Washington Sea Grant, and the College of Agriculture and Home Economics at Washington State University.

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Appendix

The SUR process developed by Zellner (1962) can be illustrated with a generalized regression equation. Suppose the ith equation in a system of m equations is

$$\mathbf{y}_i = \mathbf{X}_i\boldsymbol{\beta}_i + \boldsymbol{\mu}_i, \quad i = 1,\ldots,m$$

where $\mathbf{y}_i$ is a $n \times 1$ vector, $\mathbf{X}_i$ is a $n \times k$ matrix, $\boldsymbol{\beta}_i$ is a $k \times 1$ vector of parameters, and $\boldsymbol{\mu}_i$ is a $n \times 1$ disturbance term vector. The system of m equations may be written as

$$\begin{bmatrix} \mathbf{y}_1 \\ \mathbf{y}_2 \\ \cdot \\ \cdot \\ \cdot \\ \mathbf{y}_m \end{bmatrix} = \begin{bmatrix} \mathbf{X}_1 & & & & \\ & \mathbf{X}_2 & & & \\ & & \cdot & & \\ & & & \cdot & \\ & & & & \mathbf{X}_m \end{bmatrix} \begin{bmatrix} \boldsymbol{\beta}_1 \\ \boldsymbol{\beta}_2 \\ \cdot \\ \cdot \\ \cdot \\ \boldsymbol{\beta}_m \end{bmatrix} + \begin{bmatrix} \boldsymbol{\mu}_1 \\ \boldsymbol{\mu}_2 \\ \cdot \\ \cdot \\ \cdot \\ \boldsymbol{\mu}_m \end{bmatrix}$$

or, in matrix notation,

$$\mathbf{y} = \mathbf{X}\boldsymbol{\beta} + \boldsymbol{\mu}$$

where $\mathbf{y}$ is a $(mn) \times 1$ vector, $\mathbf{X}$ is a $(mn) \times (km)$ matrix, $\boldsymbol{\beta}$ is a $(km) \times 1$ vector, and $\boldsymbol{\mu}$ is a $(mn) \times 1$ vector.

Letting μ_{it} be the error term for the ith equation in the tth time period, the existence of contemporaneous error correlation, but no autocorrelation, implies that $E[\mu_{it}\mu_{js}] = \sigma_{ij}$ if $t = s$, but 0 if $t \neq s$. The covariance matrix for any pair of equations is $\boldsymbol{E}[\boldsymbol{\mu}_i\boldsymbol{\mu}'_j] = \sigma_{ij}\boldsymbol{I}_n$, where $\boldsymbol{I}_n$ is an $n \times n$ identity matrix. The covariance matrix for the entire system-of-equations is

$$\boldsymbol{\theta} = E[\boldsymbol{\mu}\boldsymbol{\mu}'] = \begin{bmatrix} \sigma_{11}\boldsymbol{I}_n & \sigma_{12}\boldsymbol{I}_n & \cdots & \sigma_{1m}\boldsymbol{I}_n \\ \sigma_{21}\boldsymbol{I}_n & \sigma_{22}\boldsymbol{I}_n & \cdots & \sigma_{2m}\boldsymbol{I}_n \\ \vdots & \vdots & & \\ \sigma_{m1}\boldsymbol{I}_n & \sigma_{m2}\boldsymbol{I}_n & \cdots & \sigma_{mm}\boldsymbol{I}_n \end{bmatrix} = \boldsymbol{\Sigma} \otimes \boldsymbol{I}_n.$$

The symbol $\otimes$ denotes Kronecker multiplication of matrices, and

$$\boldsymbol{\Sigma} = \begin{bmatrix} \sigma_{11} & \sigma_{12} & \cdots & \sigma_{1m} \\ \sigma_{21} & \sigma_{22} & \cdots & \sigma_{2m} \\ \vdots & \vdots & & \\ \sigma_{m1} & \sigma_{m2} & \cdots & \sigma_{mm} \end{bmatrix}$$

where the matrix $\boldsymbol{\Sigma}$ is positive definite and symmetric so that $\sigma_{ij} = \sigma_{ji}$. The generalized least squares estimate of $\boldsymbol{\beta}$, $\boldsymbol{b}$, is

$$\boldsymbol{b} = (\boldsymbol{X}'\boldsymbol{\theta}^{-1}\boldsymbol{X})^{-1}\boldsymbol{X}'\boldsymbol{\theta}^{-1}\boldsymbol{y}.$$

The problem encountered in obtaining parameter estimates from the previous equation is that $\boldsymbol{\Sigma}$ is unknown. Zellner suggested that ordinary least squares be applied to each equation to obtain the least squares residuals. These residuals are then used to obtain an estimate of $\boldsymbol{\Sigma}$, so that $\hat{\boldsymbol{\Sigma}}$ replaces $\boldsymbol{\Sigma}$ and $\hat{\boldsymbol{\theta}}$ replaces $\boldsymbol{\theta}$ in the preceding equation. In the case where $\boldsymbol{y}_i$ is a nonlinear function of $\boldsymbol{\beta}_i$, i.e. $\boldsymbol{y}_i = f_i(\boldsymbol{X}_i;\boldsymbol{\beta}_i) + \boldsymbol{\mu}_i$, for $i = 1,\ldots, m$, iterative numerical techniques are used to find the estimates of $\boldsymbol{\beta}_i$ that minimize $\hat{\boldsymbol{\mu}}'(\hat{\boldsymbol{\Sigma}} \otimes \boldsymbol{I}_n)^{-1}\hat{\boldsymbol{\mu}}$, where $\boldsymbol{\Sigma}$ is estimated from estimated residuals based on nonlinear least squares estimates of the equations in the system.

Tissue Response of Fish to Invasion by Larval *Pseudoterranova decipiens* (Nematoda; Ascaridoidea)

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Ramakrishna, N. R., and M. D. B. Burt. 1991. Tissue response of fish to invasion by larval *Pseudoterranova decipiens* (Nematoda; Ascaridoidea). Can. J. Fish. Aquat. Sci. 48: 1623–1628.

The histopathology of *Pseudoterranova decipiens* (L_3) in experimentally infected rainbow trout (*Oncorhynchus mykiss*) and naturally infected Atlantic cod (*Gadus morhua*) was similar. The chronic granulomatous inflammatory reaction included polymorphonuclear neutrophils, macrophages, lymphocytes, epithelioid cells, and fibroblasts. Giant cells were also found but only in the experimentally infected rainbow trout. Mature capsules around the larvae consisted of an inner layer, composed of macrophages which underwent epithelioid transformation and later gradually degenerated, and an outer layer, composed of fibroblasts and collagen fibres. A layer of lipofuscin was adjacent to the parasite in older cod infections but this was absent in all of the newly formed capsules in the experimentally infected rainbow trout.

L'histopathologie de *Pseudoterranova decipiens* (L_3) était similaire chez des truites arc-en-ciel (*Oncorhynchus mykiss*) infestées expérimentalement et chez des morues franches (*Gadus morhua*) infestées naturellement. La réaction inflammatoire chronique granulomateuse présentait des neutrophiles polymorphonucléaires, des macrophages, des lymphocytes, des cellules épithélioïdes et des fibroblastes. On a aussi trouvé des cellules géantes, mais seulement chez les truites arc-en-ciel infestées expérimentalement. Les capsules mûres qui entouraient les larves se composaient d'une couche interne, constituée de macrophages qui subissaient une transformation épithélioïde puis dégénéraient graduellement par la suite, et une couche externe, constituée de fibroblastes et de fibres de collagène. Une couche de lipofuscine était adjacente au parasite dans les infections anciennes chez les morues, mais elle était absente dans toutes les capsules nouvellement formées chez les truites arc-en-ciel infestées expérimentalement.

Received October 16, 1990
Accepted March 15, 1991
(JA760)

Reçu le 16 octobre 1990
Accepté le 15 mars 1991

The third-stage larvae (L_3) of the nematode *Pseudoterranova decipiens* (Nematoda; Ascaridoidea), presently known as sealworm (formerly codworm), occur in the musculature of cod and other marine fishes. The increasing abundance of this worm in the Northwest Atlantic (McClelland et al. 1987, 1990) is a serious economic problem, costing the fishing industry in Atlantic Canada alone about $30 million in 1984 (Malouf 1986). The fish host encapsulates these parasites preventing their continual migration and consequent destruction of muscle tissue. Margolis (1970) provided an excellent summary of the effects of nematodes on marine fishes; more recently, several papers relating to *P. decipiens* have been published in a collection edited by Bowen (1990). Although the pathogenicity of anisakid nematodes in liver infections is better known, comparatively little is known of the effect of sealworm, specifically, on fish muscle tissue. Earlier studies on capsule formation in the muscle (Kahl 1938; Widera 1976) were based on natural infections. In such studies, the age of any infection is not known. The objective of the present study is to understand the nature of the sequential histopathological changes in capsule formation around the larvae of *P. decipiens* in their fish host. In naturally infected cod, it is difficult to monitor the course of infection and the consequent tissue response in relation to precise age of infection. Therefore, in this study we used a laboratory-reared, uninfected experimental host, rainbow trout (*Oncorhynchus mykiss*), to study the sequential pathology and compared our results with observations on natural infections in Atlantic cod (*Gadus morhua*).

First there are some records of larvae of the marine ascaridoid *Anisakis* ("whaleworm") in wild and cultured trout (Wootten and Smith 1975). Second, in laboratory aquatic research, rainbow trout is considered as a counterpart to the white rat because of its several desirable qualities (Wolf and Rumsey 1985).

Using these fish as experimental hosts gave several advantages: (1) the precise age of each infection would be known, (2) although rainbow trout do not normally harbour marine ascaridoids, there are records of a related worm, *Anisakis simplex*, in wild and cultured rainbow trout (Wootten and Smith 1975), (3) if the fish response was similar to that of cod, then the use of such an easily controlled host with several desirable qualities (Wolf and Rumsey 1985) could be extended to other studies, and (4) it is possible to acclimate them to seawater, the natural environment in which natural infections occur.

Materials and Methods

Experimental Infection

Fish

One hundred rainbow trout (17–25 cm), 20 in each of five fish tanks, were acclimated to full-strength seawater using "Instant Ocean" artificial sea salts in a recirculation system

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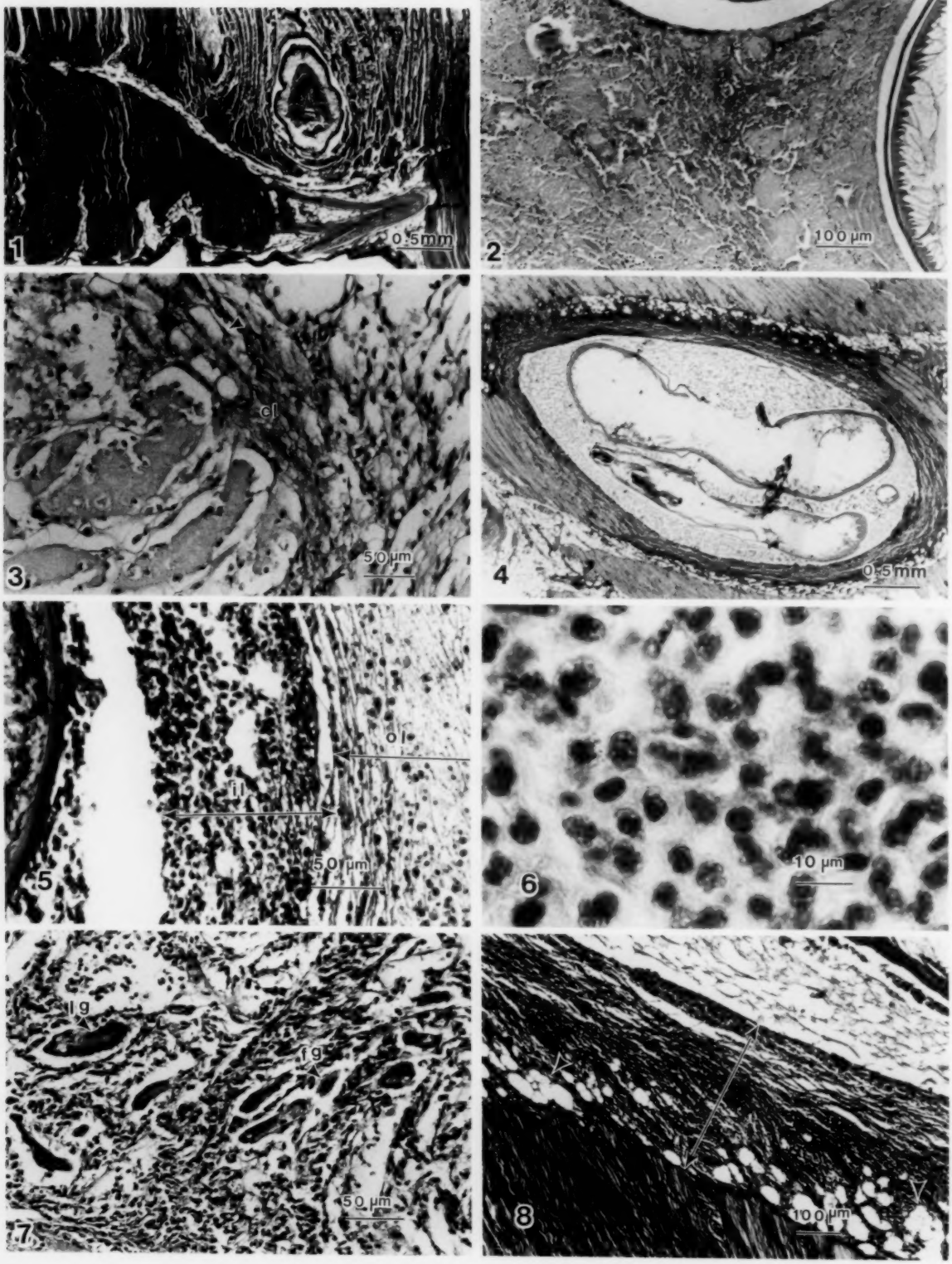
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(Landless 1976). The system was maintained in a constant temperature and photoperiod room (15°C; 12 h). The water quality was monitored for dissolved oxygen, ammonia, nitrate, and nitrite every week using HACH test kits (HACH Company, Colorado, USA).

Parasites

Infective larvae (L_3) were dissected carefully out of the musculature of naturally infected Atlantic cod, care being taken not to break the worm intestine. All larvae were identified to species and checked for any damage with a Wild M4A dissecting microscope. Viable larvae were rinsed thoroughly in 0.85% saline and kept at 4°C for no longer than 8–24 h prior to being used in experimental infections.

Infection technique

Two larvae were introduced into the stomach of anaesthetized fish with a stomach tube (6-mm inner diameter, 8-mm outer diameter) and a bulb. The anaesthetic was tertiary-amyl alcohol, and fish were intubated immediately after movement ceased.

After infection, individual fish were placed in small aquaria, where recovery was monitored. If larvae were regurgitated, the fish were reinfected. The infected fish were placed back into their respective tanks after recovery. No fish died during the infection process.

Twenty fish were necropsied at each of 2, 4, 8, 16, and 32 days postinfection (DPI). Tissues immediately surrounding the invading worm were excised and fixed.

Natural Infection

Tissue blocks of L_3 larvae with surrounding fish muscle were taken from freshly killed cod of varying sizes which were caught in Passamaquoddy Bay, New Brunswick. The tissue blocks were later classified as early, later, and old infections, based on the colour of the worm.

Early: Larvae viewed through the tissue look yellowish white to light yellow and they are not encapsulated. These are presumed to be early infections.

Later: These larvae in the tissue block were yellow to light brown and are encapsulated.

Old: These larvae in the tissue block were brown to black and are also encapsulated.

Five tissue blocks at each stage of infection were excised and fixed for light microscopical studies.

Preparation of Specimens for Light Microscopy

Tissues were fixed in Bouin's fluid and 10% phosphate-buffered formalin. Tissues were processed using standard histological techniques (Humason 1972; Pearse 1985). Paraffin wax sections 5–7 μm thick were cut using a Reichert microtome.

Stains used were haematoxylin and eosin (H & E) for general histology, Masson's trichrome for connective tissue, bleaching and Masson–Fontana for melanin, Perl's prussian blue for Haemosiderin, von Kossa method for calcium, PAS for carbohydrates, Feulgen reaction for DNA, and Huek's method for distinguishing lipofuscins from melanins.

Preparation of Specimens for Electron Microscopy

Tissues were fixed in freshly prepared Karnovsky fixative (pH 7.3) for 4–12 h at room temperature, washed in 0.1 M sodium cacodylate buffer for 30 min, postfixed in 1% osmium tetraoxide for 1 h, and washed again in 0.1 M sodium cacodylate buffer for 30 min.

For transmission electron microscopy (TEM), tissue blocks were dehydrated in a graded acetone series, infiltrated with Epon-Araldite resin, and polymerized at 45°C for 24 h and again at 60°C for 24–48 h. Gold and silver sections (50–60 nm) were cut, mounted on formvar-coated grids, stained with uranyl acetate and lead citrate, and viewed at 60 kV using a Phillips 400T transmission electron microscope. For orientation purposes, some sections from each block were cut at 1 μm, stained with tuluidine blue, and examined under a light microscope.

For scanning electron microscopy (SEM), tissue blocks were dehydrated in a graded ethanol series, critical point dried using carbon dioxide (Balzers, C.P.D.-020), coated with gold, and viewed at 10 kV in a JEOL, JSM-6400 scanning electron microscope.

Results

Experimental Infection

All larvae recovered from successfully infected rainbow trout were coiled or partially coiled at 2, 4, and 8 DPI. The majority of them were found in the musculature adjacent to the body cavity whereas at 16 and 32 DPI, larvae were found in the musculature either near the head or near the tail region.

At 2 DPI, the larvae were found lying in blood-filled cavities formed as a result of tissue destruction and damage to the blood vessels. Many larvae were found in the muscle between the connective tissue septa. The initial lesion (Fig. 1) showed muscle necrosis with loss of striations and rupture of sarcolemmae. Within necrotic tissue, there were many focal haemorrhages as well as focal and dispersed accumulations of leucocytes, predominantly polymorphonuclear neutrophils (PMN's) (Fig. 2).

At 4 DPI, the majority of the larvae were coiled or partially coiled. The area of necrosis was large. Small haemorrhages were still present. The infiltration of inflammatory cells was extensive. On this day, PMN's were still in large numbers.

At 8 DPI, there were areas of necrotic muscle with some haemorrhages. The inflammatory cellular infiltrate consisted

FIG. 1–8. *Pseudoterranova decipiens* in experimentally infected rainbow trout. Fig. 1. Histological lesion showing the parasite in the muscle with early necrosis. Masson's trichrome. Fig. 2. Light micrograph of the host cellular response at 2 DPI. H & E. Fig. 3. Section showing the proliferation of fibroblasts (arrowed) and laying of collagen (cl) at 8 DPI at the outer edge of the lesion. H & E. Fig. 4. Light micrograph of a capsule at 16 DPI showing the "granuloma." H & E. Fig. 5. Section through the capsule at 16 DPI showing an inner layer (il) consisting of macrophages/epithelioid cells and an outer layer (ol) mainly of fibroblasts and collagen. Mallory's stain. Fig. 6. Section showing epithelioid cells in the inner layer of the capsule. Note the indeterminate cell boundaries and prominant nucleolus. H & E. Fig. 7. Multinucleated Langhans type (lg) and foreign body type (fg) giant cells in the capsule at 16 DPI. H & E. Fig. 8. Mature capsule at 32 DPI. The capsule is compact and more fibrous. There is vacuolation (arrowed) at the outer edge of the capsule. H & E.

mainly of macrophages followed by PMN's and lymphocytes. The fibroblasts (Fig. 3) at the outer edge had begun to proliferate and polymerize collagen locally.

At 16 DPI, the worm had been encapsulated (Fig. 4). Much of the necrotic tissue had been replaced with inflammatory cells and connective tissue; most cells were epithelioid cells and macrophages. There were more lymphocytes than before. The capsule appeared to be compact with two distinct layers (Fig. 5). The innermost layer, adjacent to the parasite, consisted mainly of macrophages, which by 32 DPI had become epitheliod with a foamy appearance (Fig. 6); beneath these, in some specimens, many multinucleated giant cells (Fig. 7) of both "Langhans" and "foreign body" type were observed. The outermost layer consisted mainly of concentric layers of active fibroblasts laying down collagen.

By 32 DPI, the capsule was much more fibrous and compact and vacuolation was observed at the outer edge of the capsule (Fig. 8). There was progressive degeneration of the innermost layer.

Natural Infection

In the "early" infections in cod, histologically the lesion consisted of muscle necrosis with loss of striations but few inflammatory cells (Fig. 9).

In "later" infections, most larvae were encapsulated in a layered capsule. The capsule appeared to consist of two layers (Fig. 10). The innermost layer consisted of macrophages and epithelioid cells, at various stages of degeneration. The outer layer consisted of connective tissue within which two distinct zones could be noticed: a dense inner layer and a loose outer layer.

In "old" infections, the capsule consisted of only a connective tissue layer with two distinct zones: an inner, thinner zone of dense connective tissue, and an outer, thicker loose zone of connective tissue. Vascular infiltration was evident in the outer loose connective tissue. The inflammatory cells which were present earlier in the innermost layer degenerated to form a dense eosinophilic ground substance in which was embedded nuclear debris. The most interesting feature in old infections in cod was the deposition of a layer of brownish black material around the parasite (Fig. 11). Since this deposition was dark in colour, and as melanin deposition has been reported in many other parasitic infections (Roberts 1975), it was stained specifically for melanin. The result, however, was negative. Later, several histochemical reactions were performed on the pigmented layer which stained positively for lipofuscin (Table 1).

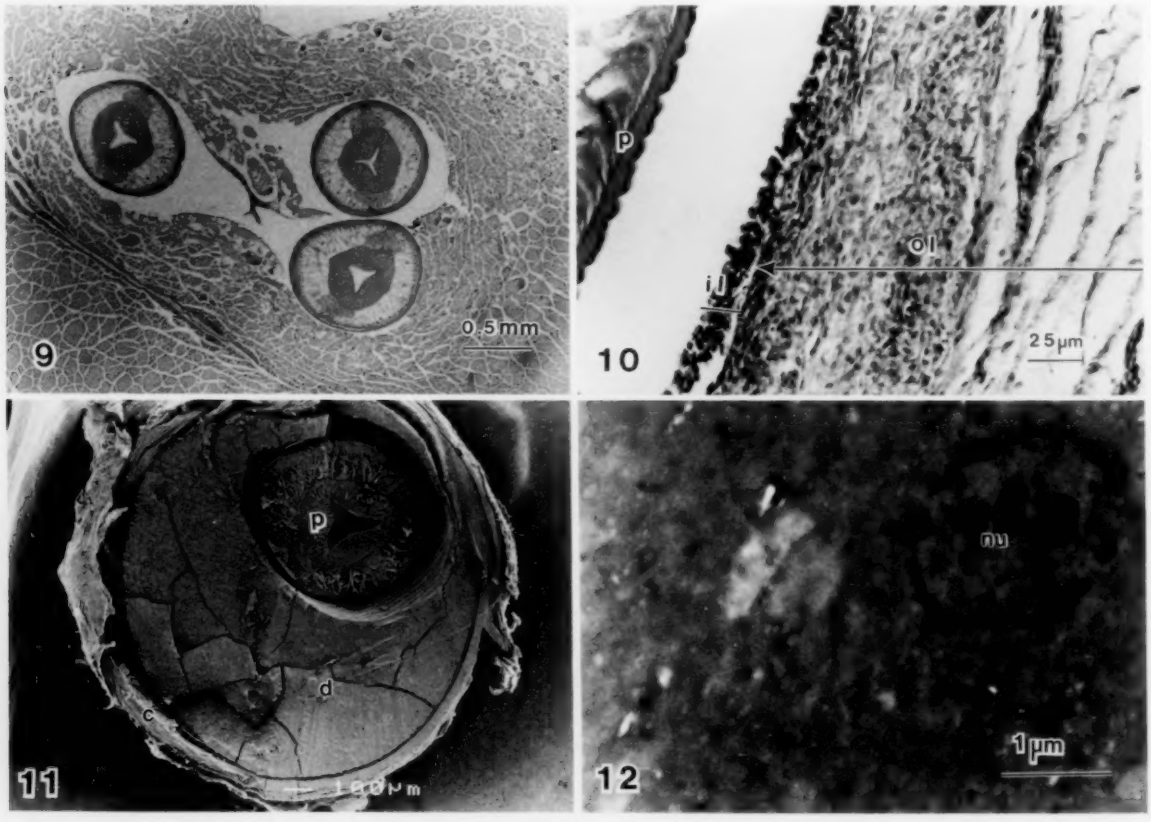

FIG. 9–12. *Pseudoterranova decipiens* in naturally infected Atlantic cod. Fig. 9. Histological section showing muscle lesion in "early" infections in cod. Fig. 10. Micrograph of a capsule in "later" infections in cod showing an inner layer (il) of inflammatory cells and an outer layer (ol) of connective tissue with two distinct zones of connective tissue: an inner dense zone and an outer loose zone. p = parasite. H & E. Fig. 11. Scanning electron micrograph of brownish black deposition (d) around the parasite (p) in the capsule (c) of "old" infections in cod. Fig. 12. Electron micrograph of a pigmented layer showing nucleii (nu) embedded in the electron-dense pigmented material.

TABLE 1. Histochemical reactions of the pigmented layer of the capsule.

Histochemical test used	Material tested for	Reaction
Bleaching in 40% peracetic acid	Melanin/lipofuscin	+
Masson–Fontana method	Melanin	–
von Kossa method	Calcium	–
Perl's blue reaction	Haemosiderin	–
PAS	Carbohydrate	+
Sudan black B	Lipids	–
Feulgen reaction	DNA	–
Huek's method	Lipofuscin	+

The electron microscopical observations of the pigmented layer reveal that the layer is heterogeneous with electron-dense material, within which are entrapped nuclei and other cellular debris (Fig. 12).

Discussion

The tissue response of rainbow trout to the presence of *P. decipiens* is typical of a chronic granulomatous inflammatory reaction. The response started with an acute inflammatory reaction; this was quickly followed by a macrophage response; the host capsule at the end of the experiment consisted of an inner layer of macrophages and epithelioid cells, with few scattered multinucleate giant cells and an outer layer of active fibroblasts and collagen. The presence of epithelioid cells, giant cells, and lymphocytes, along with macrophages, in the capsule of *P. decipiens* suggests a cell-mediated immune response.

The tissue response in cod, although not strictly comparable, appeared to be qualitatively similar to that of rainbow trout. The host capsule in cod, as in rainbow trout, consisted of an inner layer of inflammatory cells and an outer layer of connective tissue. The response in cod, however, is devoid of any initial granulocytic response which is apparent in rainbow trout where massive infiltration of PMN's marked the earliest response suggesting the involvement of granulocytes in the encapsulation process. The capsules of "later" and "old" stages of infections in cod are comparable with rainbow trout capsules at 16 and 32 DPI, respectively, in their qualitative aspects but differ in quantitative aspects, such as intensity of response and thickness of capsule. The direct comparison of experimental and natural lesions, as to the time and sequence of cellular response, is difficult, since it is not known how long the natural infections have been present.

The inflammatory response in fish has been described in response to a variety of agents including both inert materials and parasites (Prusevich 1964; Mikhailova et al. 1964; Finn 1970; Finn and Nielson 1971; Timur et al. 1977; Hauck and May 1977; Sommerville 1981; Elarifi 1982). The fibrous capsule of the present study is in general agreement with the chronic granulomatous inflammatory reaction as reported by these authors, suggesting that in fish, nonspecific inflammatory responses play a major role in defence against parasitic invasions resulting in the encapsulation of the parasite.

Multinucleate giant cells in fish are assumed to be derived by fusion of macrophages or epithelioid cells, due to similarities of chronic inflammatory response in fish to that of higher vertebrates (Richards et al. 1978; McVicar and McLay 1985). More substantial proof of this transformation, from in vitro cultures, has been provided by Secombes (1985). In the present study, giant cells were observed only in rainbow trout in the innermost layer surrounded by epithelioid cells. In many instances, epithelioid cells with two to many nuclei were seen in the lesion, suggesting that multinucleate giant cells might have been formed by the fusion of epithelioid cells.

The pigmentation of the ascaridoid nematode capsule, although reported earlier (Margolis 1970), has not been characterized. The histochemical tests performed on the brownish black deposition indicate that it is lipofuscin. This pigment is formed by the oxidation of unsaturated tissue lipids or lipoproteins, and the colour of the lipid pigments is due to the polymerization of the aldehydes produced by oxidation (Pearse 1985). Agius (1985), in his review on the melano-macrophage centres of fish, has dealt in detail with the pigmentary system of fishes. In fish, lipofuscin is by far the most widespread pigment, being found in the melano-macrophages of all species. Agius and Agbede (1984) suggested that lipofuscin is a metabolic product derived from the degeneration of cellular components, such as mitochondria, through the process of lipid mitochondrion peroxidation. The presence of entrapped nucleii in the pigmented layer (Fig. 12) suggests the degeneration of inflammatory cells, which might have contributed to the formation of lipofuscin through the lipid peroxidation of subcellular organelles.

The similarity in response of rainbow trout and cod to invasion with *P. decipiens* supports the use of rainbow trout as a model host in further work on this nematode and its population dynamics.

In summary, this study describes the sequential aspects of the inflammatory response to invading *P. decipiens* larvae and their eventual encapsulation in the muscle of fish paratenic hosts. The pigmentation within the capsule, hitherto not characterized, is found to be lipofuscin.

Acknowledgements

We are grateful to Dr. K. Aterman, Dr. Carol M. Morrison (DFO, Halifax, N.S.), and Dr. Jim Goltz (Regional Veterinary Laboratory, New Brunswick) for their help in the interpretation of slides, Dr. F. B. M. Cowan, Dr. B. M. MacKinnon, Dr. P. Sivasubramanian, and Dr. Carl Bursey for their helpful advice in histology, and Ms. Carolyn Likely, Mr. F. Purton, and the Huntsman Marine Science Centre for help in collecting worms. Financial assistance was provided by the Natural Sciences and Engineering Research Council of Canada (operating grant A2358 to M.D.B.B.) and through a contract from the Canada/New Brunswick Industrial Innovation and Technology Department Subsidiary Agreement. The senior author was supported during this study by a Commonwealth Scholarship from Canadian Commonwealth Scholarship and Fellowship Plan.

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Recurrent Depth Maxima of the Hepatotoxic Cyanobacterium *Oscillatoria agardhii*

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Toxic cyanobacteria, which have been implicated in human illness and animal mortalities, are common in many lakes, but their seasonal and vertical distribution is poorly known. We report persistent and recurrent metalimnetic blooms of *Oscillatoria agardhii* in the eutrophic Lake Östra Kyrksundet (southwest Finland) and associated toxin peaks analyzed quantitatively using liquid chromatography. Marked metalimnetic maxima of toxic *O. agardhii* developed during 1988–90. The desmethyl-microcystin-RR (a cyclic heptapeptide hepatotoxin) levels in the metalimnion were 20–40 $\mu g \cdot L^{-1}$ and paralleled the chlorophyll *a* concentrations.

Les cyanobactéries toxiques, auxquelles on impute des maladies humaines et des mortalités animales, sont communes dans de nombreux lacs, mais on connaît mal leur répartition saisonnière et verticale. Nous rapportons des proliférations persistantes et récurrentes d'*Oscillatoria agardhii* dans le métalimnion du lac eutrophe Östra Kyrksundet (sud-ouest de la Finlande), et nous avons observé des pics toxiques associés que nous avons analysés quantitativement grâce à la chromatographie en phase liquide. Des maximums marqués de prolifération d'*O. agardhii* dans le métalimnion ont été observés en 1988–1990. Les concentrations de desméthyl-microcystine-RR (hépatotoxine heptapeptide cyclique) dans le métalimnion atteignaient 20–40 $\mu g \cdot L^{-1}$ et concordaient avec les concentrations de chlorophylle *a*.

Received November 13, 1990
Accepted March 13, 1991
(JA800)

Reçu le 13 novembre 1990
Accepté le 13 mars 1991

Blooms of toxic cyanobacteria have been recorded in a large number of lakes and brackish water areas in many countries (Skulberg et al. 1984; Carmichael et al. 1985; Codd and Poon 1988; Sivonen et al. 1990). There are also reports on cattle, wildlife, and fish mortalities and cases of human illness related to toxic cyanobacteria (Skulberg et al. 1984; Carmichael et al. 1985; Gorham and Carmichael 1988; Falconer 1989). Very often, hepatotoxic heptapeptides, now called microcystins (MCYST), have been involved. Chemical analysis of cyanobacterial hepatotoxins using high-performance liquid chromatography (HPLC) was introduced during the 1980s (Siegelman et al. 1984; Gathercole and Thiel 1987; Harada et al. 1988; Meriluoto and Eriksson 1988).

Effects of toxic cyanobacteria on zooplankton (Lampert 1981; Nizan et al. 1986; Fulton and Paerl 1987) and accumulation of cyanobacterial peptide toxins in mussels have also been reported from aquarium experiments (Eriksson et al. 1986, 1989a) and from field studies (Lindholm et al. 1989a, 1989b). Although toxic cyanobacteria occur in a great number of lakes, quantitative data on seasonal and vertical distribution of cyanobacterial toxins are very sparse (Lindholm et al. 1989b, 1992).

Some strains of the filamentous cyanobacterium *Oscillatoria agardhii* Gom. produce microcystins (Berg et al. 1988; Meriluoto et al. 1989), and this organism has caused problems in a number of Scandinavian lakes and reservoirs (Berg et al. 1986; Lindholm et al. 1989b; Sivonen et al. 1990). The aim of this study was to investigate the distribution of *O. agardhii* and its peptide toxins in a eutrophic lake (Lake Östra Kyrksundet) in relation to hydrographic conditions and to assess the public health implications of our results.

Material and Methods

Lake Östra Kyrksundet is a eutrophic lake (200 ha, maximum depth 22 m, mean depth 8.5 m) situated on Åland Island, southwest Finland (Fig. 1). The drainage area (38 km^2) consists of farmland, mixed forests, several small lakes, and areas with bare "rapakivi" granite rocks (Lindholm 1975; Lindholm et al. 1989b). The theoretical water renewal time is about 2 yr. The lake is relatively sheltered from winds, and thermal stratification lasts from early May to late September or the beginning of October. Ice cover usually lasts from December to late April. In 1989 and 1990 the winter was mild and the ice disappeared in March. The early summer periods May–July of 1988 and 1989 were warm and sunny and August was rainy in both years.

Water samples were taken with a Ruttner sampler at 1-m depth intervals. In 1989 and 1990, 0.5-m intervals were used for the metalimnion. Hydrographical parameters were measured as in Lindholm et al. (1989b). Unfixed water samples were examined at 200× and 400× with an inverted microscope. Samples for chlorophyll *a* (300 mL) and toxin analyses (200 mL) were filtered on Whatman GF/C filters (diameter 47 and 25 mm, respectively), air-dried for about 1 h, and stored frozen at −20°C.

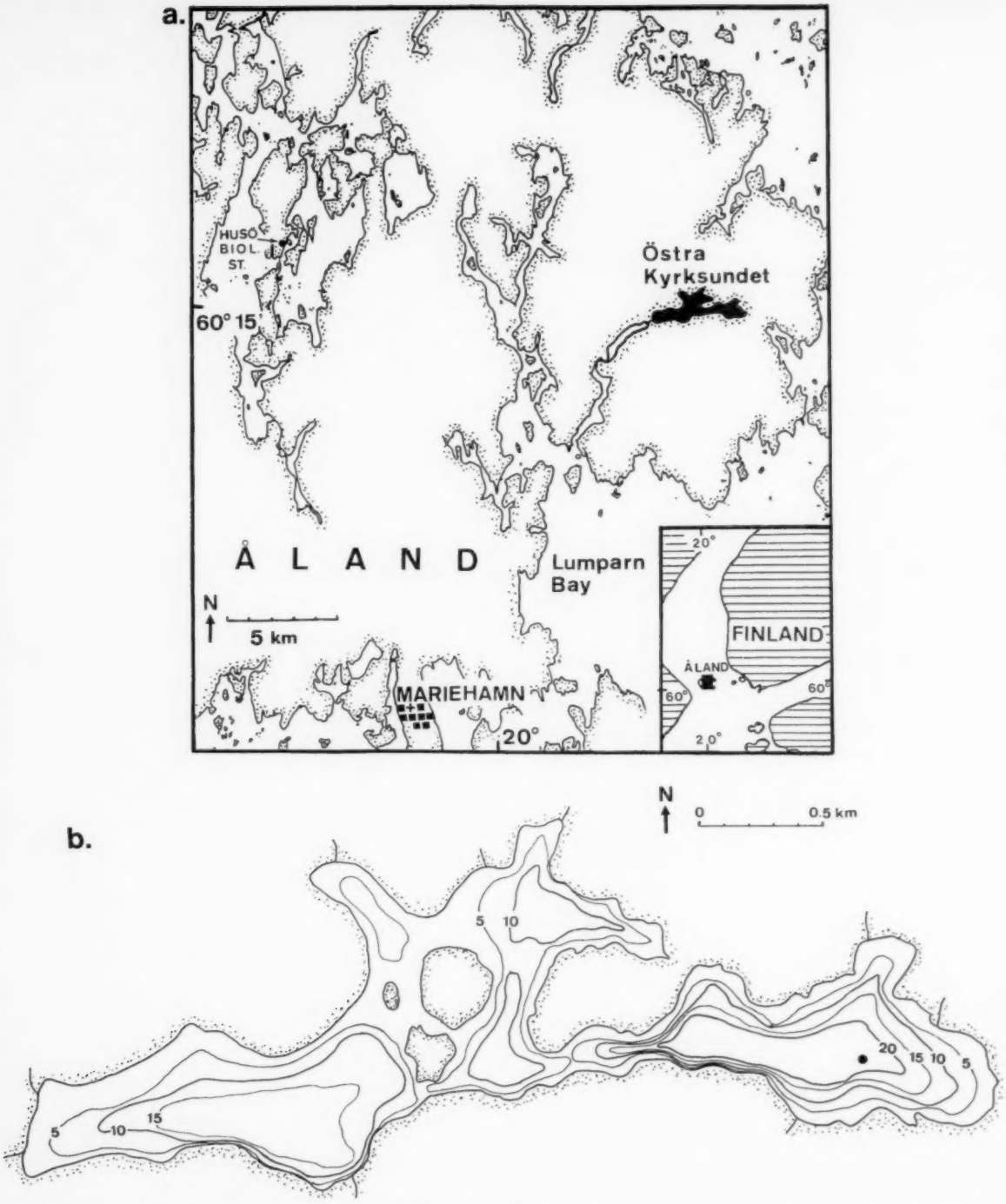

FIG. 1. (a) Map showing the location of Lake Östra Kyrksundet on Åland Island, southwest Finland. (b) Bathymetric map of Lake Östra Kyrksundet. The solid circle marks the sampling site.

The toxin analyses were performed with the internal surface reversed-phase HPLC method described in Meriluoto and Eriksson (1988) and Meriluoto et al. (1990a, 1990b). The GF/C filters were extracted twice using 5 min of ultrasonication with 1.0 mL of 15% acetonitrile – 85% 0.1 M KH_2PO_4, pH 6.8, or, alternatively, with 1.0 mL of water followed by 1.0 mL water–methanol–butanol (75:20:5, by volume). The extracts were filtered through Gelman (Ann Arbor, MI) Acro

LC13 0.45-μm filters and injected in the HPLC. The column was a Regis (Morton Grove, IL) GFF column which was eluted at 1.0 mL·min^{-1} with 15% acetonitrile – 85% 0.1 M KH_2PO_4, pH 6.8 (250 × 4.6 mm column) or with 12% acetonitrile – 88% 0.1 M KH_2PO_4, pH 6.8 (150 × 4.6 mm column). The eluate was monitored at 238 nm.

Chlorophyll *a* was measured spectrophotometrically after homogenization and 2 h of extraction in 15 mL of 90% acetone at room temperature (Marker et al. 1980). Total phosphorus (TP) and total nitrogen (TN) were analyzed according to Koroleff (1979) on samples taken at 1- or 5-m depth intervals.

Results

Occurrence of *Oscillatoria*

Marked metalimnetic maxima of *O. agardhii* and chlorophyll *a* developed in both 1988 and 1989 (Fig. 2). During June and July 1989, *O. agardhii* formed an almost monospecific bloom at 6–8 m. In July 1990, *O. agardhii* was abundant in both the metalimnion (chlorophyll *a* value over 40 μg·L^{-1}) and the hypolimnion (Fig. 2c). There was also good agreement between chlorophyll *a* distribution and distribution of *O. agardhii* toxin (desmethyl-microcystin-RR) in the metalimnion in all three summers (Fig. 2).

In the *Oscillatoria* layer, often just below the toxin maximum, a marked oxygen minimum developed (Fig. 2). In 1988 and 1989 the metalimnetic *O. agardhii* population declined in late July before the vertical mixing reached the metalimnion. At intervals there were slight increases in the epilimnetic chlorophyll *a* and toxin concentrations, indicating that part of the deep *O. agardhii* population entered the mixed layer. In all three summers the Secchi depth was between 2 and 3 m. On sunny days the light level of the upper metalimnion (6 m) was 5–15 μE·m^{-2}·s^{-1}, which is less than 1% of full daylight.

Desmethyl-microcystin-RR toxins (eluted from the HPLC column slightly before microcystin-RR at pH 6.8) were recorded in Lake Östra Kyrksundet in 1987 (Lindholm et al. 1989b), 1988, 1989, and 1990. In the summers of 1988 and 1989, when more frequent sampling was done, fairly high toxin levels occurred from the end of May and lasted to late July (Fig. 3). The highest toxin levels in the metalimnion were 20–30 μg·L^{-1} in the summers of 1988–90.

Nutrient Levels

The TP level in the epilimnion of the lake was about 30 μg·L^{-1} in 1987 (Lindholm et al. 1989b). In 1988 and 1989 the values were slightly lower, but higher TP levels were observed in the metalimnion and near the bottom in anoxic water (Table 1). The TN values were in the range 500–1000 μg·L^{-1} in the epilimnion (Table 1). The TN:TP ratio was relatively high (20–30) in the epilimnion but lower (about 15) in the metalimnion.

Discussion

Toxic *Oscillatoria* in Lake Östra Kyrksundet

Surface blooms of various cyanobacteria and metalimnetic maxima of *Oscillatoria* sp. were observed in Lake Östra Kyrksundet in 1972 and 1973 which indicated eutrophy already at that time (Lindholm 1975). Toxicity of *Oscillatoria* blooms in Lake Östra Kyrksundet was first tested and confirmed in 1987, when the cyanobacterium was distributed in both the epilimnion and the metalimnion (Lindholm et al. 1989b). In the present study, which includes novel quantitative HPLC analysis of toxins, we report persistent and recurrent metalimnetic maxima of hepatotoxic *O. agardhii*. Depth maxima, which could escape detection in routine investigations, constitute an unrecognized health risk in water supplies.

Lake Östra Kyrksundet has been abandoned as a drinking water supply since August 1987. Because the blooms persisted, purification was insufficient, and the intake of the drinking water treatment plant was situated near the metalimnion, the lake was considered unsafe as a raw water supply. Moreover, neurotoxic cyanobacteria have been recorded in the lake (Sivonen et al. 1989).

Oscillatoria agardhii and other species of cyanobacteria which may be toxic occur in the metalimnion of lakes worldwide (e.g. Watanabe 1979; Walsby et al. 1983; Reynolds 1984; Klemer et al. 1988; Konopoka 1989). Cyanobacterial blooms are usually considered to be a problem of the surface layers of heavily eutrophic lakes (Secchi depth usually less than 1 m) (Reynolds and Walsby 1975). Depth maxima of *O. agardhii*, however, tend to develop in lakes with relatively clear water (Secchi depth over 2 m) (Reynolds 1984; Lindholm 1991). The toxic depth maxima presented here are certainly not unique.

Metalimnetic populations of cyanobacteria may persist for months, but they may also be suddenly mixed into the epilimnion and deteriorate its water quality, e.g. during storms or at the onset of the autumnal overturn. Moreover, *Oscillatoria* filaments rise towards the surface due to buoyancy changes, e.g. on cloudy days or due to nutrient conditions (Klemer et al. 1982; Walsby et al. 1983). Thus, even a compressed depth maximum of cyanobacteria may turn into a surface bloom (Lindholm 1991).

Hygienic Aspects

Microcystins are potent hepatotoxins with intraperitoneal LD_{50} values ranging from 50 to 1000 μg·kg^{-1} in white mice (Carmichael et al. 1990). There is growing evidence that toxins from cyanobacteria may cause human illness (Carmichael et al. 1985; Gorham and Carmichael 1988). Extracts of toxic cyanobacteria have long-term and tumour-promoting effects (Falconer et al. 1988; Falconer and Buckley 1989; MacKintosh et al. 1990).

There are so far no international safety limits concerning acceptable toxin concentrations in drinking water and raw water. On the basis of recent studies of the cell toxicity and possible mutagenic effects of cyanobacterial toxins (e.g. Collins et al. 1981; Eriksson et al. 1989b; Falconer and Buckley 1989; Repavich et al. 1990; MacKintosh et al. 1990), we believe that the limits should be set extremely low.

Monitoring of Toxic Blooms

Versatile methods for toxin analysis are becoming available (e.g. Harada et al. 1988; Meriluoto and Eriksson 1988; Meriluoto et al. 1990a, 1990b). Chemical or biological toxin analyses are, however, not always accessible. Chlorophyll *a*, which is a useful indicator of the biomass, correlated with the toxin levels in Lake Östra Kyrksundet in this study. Similar results were obained in 1987 (Lindholm et al. 1989b). We do not know, however, if our findings can be extended to other monospecific

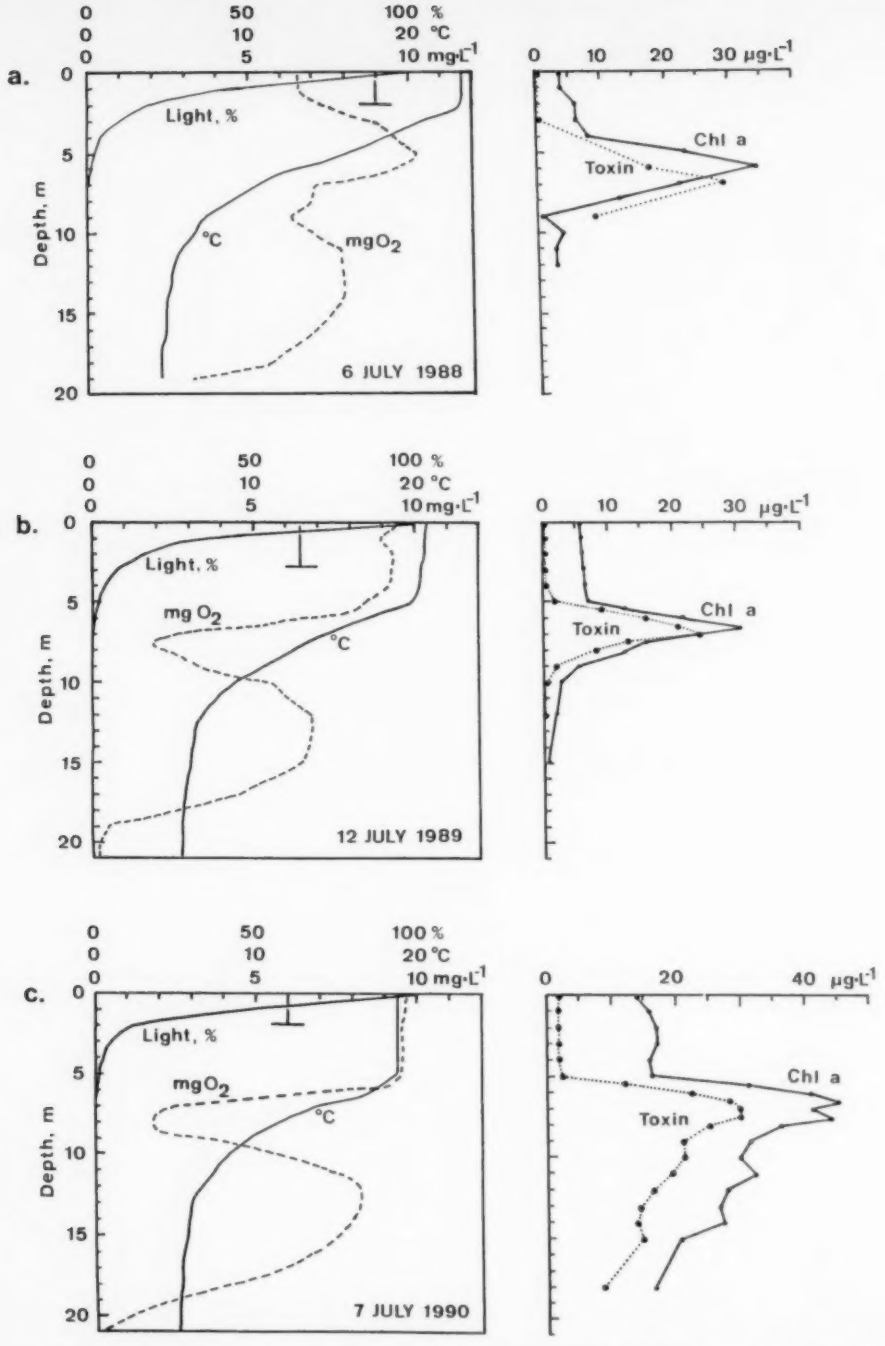

FIG. 2. Vertical distribution of chlorophyll *a* and *O. agardhii* toxin (desmethyl-microcystin-RR) in Lake Östra Kyrksundet in relation to hydrographical conditions on (a) 6 July 1988, (b) 12 July 1989, and (c) 7 July 1990. Light is a percentage of surface light, Secchi depth is indicated with a bar, and dissolved oxygen is in $mg \cdot L^{-1}$. Chlorophyll *a* and *O. agardhii* toxin are in $\mu g \cdot L^{-1}$.

cyanobacterial blooms. Toxin production probably varies in different environments, but generally, high nutrient levels make high toxin production possible (Sivonen 1990). Much higher toxin levels than we have recorded may thus occur in other lakes.

Cyanobacterial toxins occur both inside cells and in the water phase. Our convenient filter method measures toxins in particulate matter, and toxins in the water phase are not accounted for. In July 1988, we concentrated and analyzed a number of parallel samples using the rotary evaporation method (which

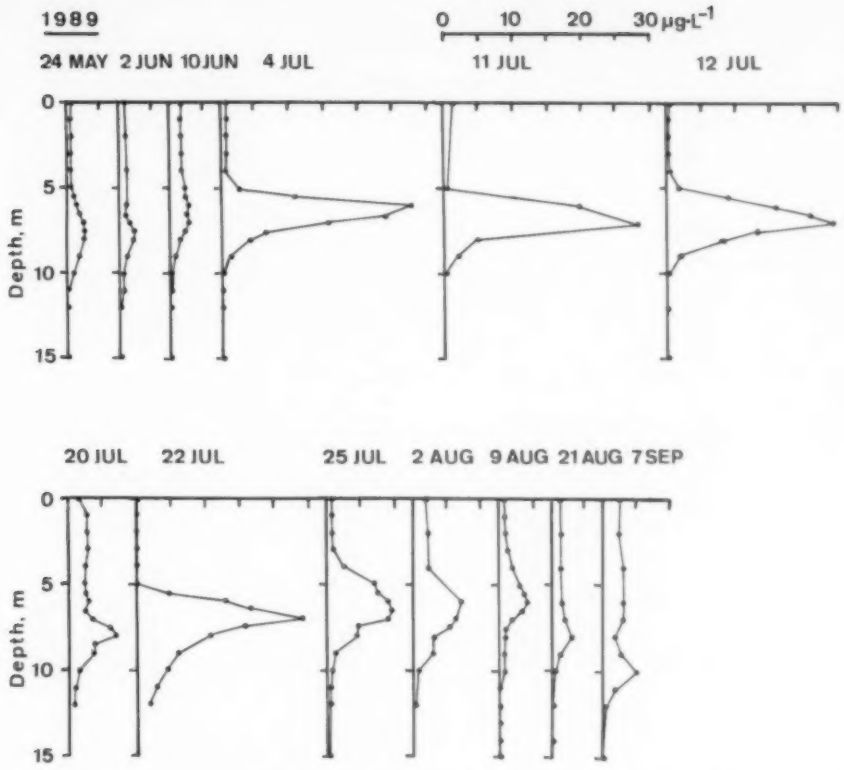

FIG. 3. Vertical and temporal distribution of *O. agardhii* toxin (desmethyl-microcystin-RR; $\mu g \cdot L^{-1}$) in Lake Östra Kyrksundet during the summer of 1989.

TABLE 1. Concentrations ($\mu g \cdot L^{-1}$) of total phosphorus (TP) and total nitrogen (TN) in Lake Östra Kyrksundet on 6 July 1988 and 12 July 1989.

Depth (m)	6 July 1988		12 July 1989	
	TP	TN	TP	TN
0	25	604	21.9	519
5	31	693	27.0	544
6			51.4	807
7			47.7	876
8			32.1	777
10	52	1258	22.2	752
15	59	1153	19.9	805
20	189	1533	109.2	1462

measures both the intracellular and the free toxin; Lindholm et al. 1989b) and the filter method (as in this study). The methods gave almost identical results, and in some cases, the toxin yield was even better in the filter method, probably due to the simpler procedure. Our findings corroborate the general view that microcystins are not released into the water phase during active growth but only when the cells lyse (Berg et al. 1987; Codd et al. 1989). Low concentrations of released cyanobacterial peptide toxins seem to be degraded by microorganisms (Berg et al. 1987).

Frequent microscopic examination would be a reasonable way of monitoring and a substitute to mouse bioassays. If well-known hepatotoxin-producing genera (e.g. *Microcystis*, *Anabaena*, and *Oscillatoria*) are observed in significant amounts in a drinking water supply, samples should be tested for toxicity. In a Finnish survey of eutrophic lakes, about 50% of the tested cyanobacterial blooms were found to be toxic (Sivonen et al. 1990). In a recent American study, about 25% of the blooms were toxic (Repavich et al. 1990). Rich occurrence of cyanobacteria in drinking water supplies should therefore always be considered a potential health hazard. The purification of raw water loaded with toxic cyanobacteria is both expensive and difficult (Falconer et al. 1989).

Quantitative and comparative studies concerning the temporal and spatial distribution of toxic cyanobacteria in lakes and reservoirs are necessary. Thorough field studies would improve the possibilities of predicting the coming of noxious blooms and evaluating the risks for humans, domestic animals, and wildlife.

Acknowledgements

This study was supported by grants from the Academy of Finland (Research Council for Environmental Sciences) and the Maj and Tor Nessling Foundation.

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Recovery of Crustacean Zooplankton Species Richness in Sudbury Area Lakes following Water Quality Improvements

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Keller, W., and N. D. Yan. 1991. Recovery of crustacean zooplankton species richness in Sudbury area lakes following water quality improvements. Can. J. Fish. Aquat. Sci. 48: 1635–1644.

Temporal patterns in the species richness of crustacean zooplankton communities were assessed in eight Sudbury area lakes based on data collected between 1973 and 1986. Excluding a consistently nonacidic reference lake, the study lakes showed general reductions in acidity and trace metal concentrations during this period, related to reduced contaminant emissions from the Sudbury smelters. Despite water quality improvements, several of the study lakes continue to have low pH and elevated trace metal concentrations which have inhibited recovery of zooplankton species richness. However, in lakes with more favourable current pH and lower trace metal concentrations, substantial increases in the average species richness of crustacean plankton communities have occurred, apparently due to both invasion of new species and more frequent occurrence of existing species. These results clearly demonstrate that reductions in acid-forming emissions lead to both chemical and biological improvements in some aquatic systems.

Les auteurs ont évalué les régimes temporaux de la richesse des espèces composant les communautés de crustacés zooplanctoniques de huit lacs de la région de Sudbury en se basant sur des données recueillies de 1973 à 1986. Sauf pour un lac de référence régulièrement non acidifié, les lacs expérimentaux ont montré des baisses générales de l'acidité et des teneurs en oligo-éléments pendant cette période, baisses liées à une réduction des émissions de polluants venant des fonderies de Sudbury. Malgré des améliorations de la qualité de l'eau, plusieurs des lacs expérimentaux montrent toujours un faible pH et des teneurs élevées en oligo-éléments qui ont gêné le rétablissement de la diversité des espèces de zooplancton. Par contre, dans les lacs de pH actuel plus approprié et à teneurs en oligo-éléments moins élevées, les auteurs ont noté des augmentations importantes de la diversité moyenne des espèces de crustacés planctoniques. Selon toute apparence, ces augmentations seraient le résultat de l'arrivée de nouvelles espèces et de la présence plus fréquente des espèces déjà sur place. Les résultats obtenus prouvent qu'une réduction des émissions acidogènes amène des améliorations de certains systèmes aquatiques autant au niveau biologique que chimique.

Received January 29, 1990
Accepted March 13, 1991
(JA452)

Reçu le 29 janvier 1990
Accepté le 13 mars 1991

Historically, severe degradation of lake water quality, related to atmospheric emissions of S and metals from local Cu and Ni smelting operations, has occurred in the Sudbury, Ontario (Canada), area (Gorham and Gordon 1960; Beamish and Harvey 1972; Dillon et al. 1979; Keller et al. 1980). While many Sudbury area lakes still remain highly acidic and metal contaminated, general water quality improvements in the region have occurred in the last decade because of substantial reductions in S and metal emissions from the smelting industry (LaZerte and Dillon 1984; Dillon et al. 1986; Hutchinson and Havas 1986; Keller and Pitblado 1986).

Observed water quality changes have often been dramatic; however, the extent of biological responses to improved water quality in Sudbury area lakes is not known. Surveys have revealed positive responses in the rotifer community (MacIsaac et al. 1986) and chrysophyte assemblages (Dixit et al. 1989) of Swan Lake and lake trout (*Salvelinus namaycush*) and zoobenthos in Whitepine Lake (Gunn and Keller 1990), but the responses of other aquatic communities have not yet been described. Despite overall reductions in S deposition across North America (Hedin et al. 1987; Dillon et al. 1988a), documentation of natural biological recovery from other areas is also very rare, restricted to observations by Kelso and Jeffries (1988) of limited reinvasion by fish in two Algoma, Ontario, lakes. Experimentally, partial recovery of the zooplankton community in artificially acidified Lake 223 was observed as the lake pH was allowed to increase from ~5.0 to ~5.5 (Chang and Malley 1989). Documentation of positive biological responses is essential to demonstrating the benefits and assessing the adequacy of emission control strategies.

In this paper we examine temporal changes in the numerical species richness (number of species in a defined number of individuals, after Hurlbert 1971) of crustacean plankton communities in seven Sudbury area lakes for evidence of recovery associated with water quality improvements, and we evaluate the factors responsible. Both direct chemical effects and indirect effects by altered predatory and competitive interactions may be expected to influence zooplankton communities (Brett 1989). Our measure of species richness is the average number of species appearing in individual collections over the ice-free

period. Recovery is assessed through comparison with the species richness of one consistently near-neutral (pH 6.9) Sudbury area lake and 24 near-neutral (pH > 6.0) Dorset, Ontario, area lakes. Species richness was chosen for examination because it is a parameter generally regarded as a measure of community health and that has shown positive relationships to pH in numerous previous synoptic zooplankton surveys in North America (e.g. Sprules 1975; Confer et al. 1983; Brezonik et al. 1984; Keller and Pitblado 1984) and Scandinavia (e.g. Almer et al. 1974; Hobaek and Raddum 1980).

Study Lakes

The Sudbury lakes range widely in size, depth, and distance from the smelters (Table 1). The nutrient status of the lakes is variable; however, all the lakes have low to moderate nutrient concentrations (total phosphorus (TP) ≤12 $\mu g \cdot L^{-1}$; total nitrogen (TN) ≤318 $\mu g \cdot L^{-1}$). With the exception of Welcome Lake, a consistently nonacidic reference lake, the Sudbury lakes had varying degrees of initial water quality impact and have exhibited increased pH and reduced Al concentrations with time (Table 2). This is attributable to decreased recent emissions of acids and acid precursors from the Sudbury smelters. The lakes closest to Sudbury have also shown substantial temporal reductions in concentrations of Cu and Ni (Table 2), metals abundant in the smelter emissions. These patterns are in agreement with the results of Dillon et al. (1986), Hutchinson and Havas (1986), and Keller and Pitblado (1986).

None of the study lakes have been directly subjected to experimental manipulations of water chemistry. Although a substantial decline in acidity had already occurred in Laundrie Lake by 1983 (pH 5.2; Keller et al. 1986), the current pH (5.6) of Laundrie Lake must, however, partially reflect the experimental neutralization of Bowland Lake, directly upstream in 1983 (Molot et al. 1986).

The 24 Dorset reference lakes (pH > 6.0) are located about 200 km southeast of Sudbury and are zoogeographically similar to the Sudbury lakes with respect to crustacean zooplankton (Sprules 1977; Keller and Pitblado 1989). They exhibit comparable morphometry to the Sudbury lakes (A_0 5.4– 679 ha; $\bar{Z}$ = 3.5–22 m; Z_{max} = 7.5–61 m) but have a wider range in nutrient status (TP = 4–45 $\mu g \cdot L^{-1}$; TN = 177–627 $\mu g \cdot L^{-1}$). Concentrations of Cu and Ni are uniformly much lower (<2 $\mu g \cdot L^{-1}$) in the Dorset lakes than in the Sudbury lakes. Total Al concentrations range from 11 to 85 $\mu g \cdot L^{-1}$. Additional background data on the Dorset lakes are provided in Reid and Girard (1987a, 1987b) and Dillon et al. (1988b).

Methods

Because historical species richness data were assembled from several independent studies, zooplankton sampling methods varied among lakes. However, with the exception of Clearwater Lake, the same technique, either vertical net hauls or Plexiglas trap (Schindler 1969) collections, was employed within individual lakes (Table 3). Yan and Strus (1980) showed no influence due to the method change in Clearwater Lake between 1975 and 1976 (nonmetered tow nets to Plexiglas traps).

Tow net collections from the Dorset lakes and from Clearwater and Whitepine lakes from 1982 on involved pooling a series of hauls from predetermined depths to generate a single composite sample corrected for lake morphometry. All other tow net samples were taken as single vertical hauls from 1 m above bottom to surface at central locations in each lake or lake basin. The species richness of simultaneously collected single vertical net hauls and multiple, volume-weighted hauls from Whitepine Lake (n = 12) was not significantly different (paired t-test; $p > 0.05$).

Samples were generally collected on a monthly or twice-monthly schedule from May to October. These two protocols give virtually identical species richness data (Yan and Welbourne 1990). Monthly averages were generated from twice-monthly collections and these were used to calculate annual averages.

All samples were preserved with 4% formalin in the field for subsequent identification. While counting effort varied substantially within this study, usually a minimum of 250 crustaceans were enumerated in each sample except in samples with fewer individuals. Copepodids and nauplii were included in the enumeration but were not identified. Overall, the average number of animals counted per sample for individual Sudbury lakes ranged from 284 to 545.

Because species richness was related to the number of animals counted, richness values were adjusted to reflect a standard counting effort of 250 animals. For correction, we derived for each lake the model of best fit of either a linear or power relationship (Table 4) between number of species and number of animals counted. Joe Lake data were not corrected because a spurious negative relationship was observed due to very high numbers of copepodids counted in the earliest study year, when

TABLE 1. Locations and selected physical and chemical characteristics of the Sudbury study lakes. Average total phosphorus (TP) and total nitrogen (TN) values are based on data on file with the Ontario Ministry of the Environment, Limnology Section and Northeastern Region, for the period 1981–86. Lakes are arranged in order of increasing distance from Sudbury.

Lake	Lat.	Long.	Distance from Sudbury (km)	A_0 (ha)	$\bar{Z}$ (m)	Z_{max} (m)	TP ($\mu g \cdot L^{-1}$)	TN ($\mu g \cdot L^{-1}$)
Clearwater	46°22′	81°03′	13	77	8.3	21.5	5	193
Swan	46°22′	81°04′	15	6	2.8	8.8	11	318
Wavy	46°18′	81°06′	21	255	10.1	34.0	4	203
Joe	46°44′	81°01′	28	180	11.2	34.0	4	183
Sans Chambre	46°43′	81°09′	29	15	5.6	15.0	12	268
Laundrie	47°07′	80°52′	80	364	4.9	20.4	7	189
Whitepine	47°17′	80°50′	89	67	5.9	22.0	6	206
Welcome	47°13′	81°02′	97	670	7.4	27.4	8	254

TABLE 2. Average concentrations of trace metals and pH in the Sudbury study lakes. Data are usually based on monthly or twice-monthly collections during the ice-free season from surveys conducted by the Ontario Ministry of the Environment, Limnology Section and Northeastern Region. Methods of data collection for the Sudbury and Dorset study lakes are outlined in MOE (1982), Locke and Scott (1986), and Keller and Pitblado (1986). Analytical methods are summarized in MOE (1981). Lakes are arranged in order of generally increasing recent pH.

Lake	Year	pH	Cu ($\mu g \cdot L^{-1}$)	Ni ($\mu g \cdot L^{-1}$)	Al ($\mu g \cdot L^{-1}$)
Clearwater[a]	1973[b]	4.3	98	283	420
	1974[b]	4.2	101	289	516
	1975	4.3	100	272	447
	1976	4.2	91	271	397
	1977	4.1	81	278	381
	1978	4.4	74	257	324
	1979	4.4	60	220	272
	1980	4.5	64	198	266
	1981	4.5	52	230	233
	1982	4.5	56	199	176
	1983	4.6	49	226	221
	1984	4.7	—	—	184
Wavy[b]	1974	4.4	33	101	—
	1985	4.7	18[c,e]	83[c,e]	208
	1986	4.7	21[c,e]	79[c,e]	198
Swan[a]	1977	4.0	64	301	289
	1982	4.8	17	82	76
	1983	5.0	12	80	67
	1984	5.1	9	58	47
Laundrie[b]	1975	4.9	3	5	—
	1976	4.8	2	4	118
	1985	5.4	1[c,e]	3[c,e]	81
	1986	5.6	2[c,e]	3[c,e]	79
Whitepine[d]	1980[c]	5.5	1	4	69
	1981[c]	5.7	2	2	59
	1982	5.8	2	2	44
	1983	5.8	1[a]	2[a]	36[a]
	1984	5.8	1	1	40
	1985	5.8	1	2	50
	1986	5.9	1	2	34
Sans Chambre[c]	1981	5.4	4	21	101
	1983	5.8	5	11	67
	1985	5.9	3	10	50
Joe[a]	1975	5.7	20	23	81
	1976	5.6	17	25	58
	1985	6.3	—	—	24
	1986	6.3	4[c,e]	10[c,e]	14
Welcome[b]	1975	6.9	3	4	—
	1976	6.7	3	2	27
	1985	6.9	—	—	24
	1986	6.9	<1[c,e]	1[c,e]	25

[a]Whole-lake, volume-weighted samples.
[b]Surface samples.
[c]Only one sampling date; 1987 data used for Joe Lake.
[d]Average of discrete epilimnion and metalimnion samples.
[e]Epilimnion/metalimnion non-volume-weighted tube composite samples.

species richness was low. No correction was applied to the Dorset lakes because close to 250 animals were counted in all the samples, and over the narrow range of counting effort, species richness and numbers counted were not correlated.

Three different taxonomists identified species in the samples. A few apparent discrepancies in species identifications were accounted for by reporting *Diaphanosoma* and *Eubosmina* at the generic level and by combining *Daphnia catawba* with *Daphnia pulex* after Dodson (1981). A comparison (paired *t*-tests) of results between taxonomists A and B on the same samples from Welcome ($n = 10$), Laundrie ($n = 8$), and Wavy ($n = 10$) lakes did not show any significant ($p > 0.05$) differences in the number of species reported. No similar comparisons were made with taxonomist C; however, except for a few very scarce species, taxa lists for consecutive sampling years (Clearwater Lake, 1975 and 1976; Joe Lake 1975 and 1976) by taxonomists A and C were identical.

Chemical and morphometric data, except pH, were $\log_{10}$ transformed prior to use in statistical tests. To assess the overall influence of correction for counting effort on patterns in the average, ice-free period species richness data, statistical tests were run on both adjusted and original species richness values.

Results and Discussion

Patterns in Species Richness

Numerous investigations have shown negative relationships between the species richness of crustacean zooplankton communities and acidity and metal contamination, related to atmospheric deposition (Sprules 1975; Yan and Strus 1980; Confer et al. 1983; Keller and Pitblado 1984). Our study demonstrates that crustacean species richness is also a sensitive measure of recovery from acidic/metal contaminated conditions.

Over the periods of study, average annual ice-free period crustacean species richness ranged from 3.2 to 12.5 (2.8 to 11.2 after correction for counting effort) species per collection in the eight Sudbury lakes and from 7.7 to 12.5 species per collection in the Dorset lakes. The average species richness in the Dorset lakes of 10.1 species per collection is almost identical to the value of 10.4 species reported by Sprules (1975) for 10 nonacidic (pH > 6.0) Sudbury area lakes. Overall, corrected and uncorrected species richness values were very similar (Table 5; Fig. 1).

In the Sudbury lakes, average annual species richness was positively correlated ($p < 0.05$) with pH ($r = 0.84$) and negatively correlated with concentrations of Al, Cu, and Ni ($r = -0.78$ to -0.82). Concentrations of Al, Cu, and Ni were negatively correlated ($p < 0.05$) with pH ($r = -0.80$ to -0.85) in the Sudbury lakes. Because of the close correlation between acidity and trace metal concentrations in the Sudbury lakes, it is not possible to separate the independent roles of these factors as they affect species richness. Certainly, however, biological impacts must represent the combined effect of acidity and trace metals in lakes close to Sudbury. In the near-neutral Dorset lakes, linear correlation analysis of annual average species richness versus chemical and physical lake characteristics revealed no significant relationships ($r < 0.32$; $p > 0.05$) between species richness and the variables tested, including A_0, Z_{max}, $\bar{Z}$, Secchi transparency, TP, TN, pH, dissolved organic carbon (DOC), and trace metals.

The corrected species richness values increased significantly over time in four of the Sudbury lakes, Wavy, Laundrie, Sans Chambre, and Joe (ANOVA, $p < 0.01$; Table 5). Average changes ranged from 1.6 to 2.9 species per collection, from comparison of early and recent sampling years. The rate of

TABLE 3. Summary of zooplankton collection methods for the study lakes. Collections were conducted on a twice-monthly or monthly basis during the ice-free season. Lakes are arranged in order of increasing distance from Sudbury.

Lake	No. of stations	Period(s)	Gear type[a]
Clearwater	4	1973–79 1981–84	80-μm mesh nonmetered tow nets (11–29.3 cm in diameter) from 1973 to 1975; 30-μm mesh Plexiglas traps (30–34.4 L) from 1976 to 1981; 80-μm mesh metered tow nets (12.5 cm in diameter) from 1982 to 1984
Swan	3	1977 1982–84	31.9- to 34-L Plexiglas traps; 80-μm mesh in 1977; 30-μm mesh in other years
Wavy	3	1974 1985–86	80-μm mesh tow nets (12.5–20 cm in diameter); metered in 1985 and 1986
Joe	3	1975–76 1985–86	23- to 34.4-L Plexiglas traps; 80-μm mesh in 1975; 30-μm mesh in other years
Sans Chambre	1	1981, 1983, 1985	80-μm mesh tow nets (12.5 cm in diameter); metered from 1983 on
Laundrie	2	1975–76 1985–86	80-μm mesh tow nets (20–20.3 cm in diameter); metered in 1985 and 1986
Whitepine	1	1980–86	80-μm mesh tow nets (12.5 cm in diameter); metered from 1982 on
Welcome	3	1975–76 1985–86	80-μm mesh tow nets (20–20.3 cm in diameter); metered in 1985 and 1986
Dorset lakes	1	1983	80-μm mesh metered tow nets (12.5 cm in diameter)

[a]Aliquots from Plexiglas trap samples from different stations and discrete depths were pooled proportionally to lake morphometry to yield a single composite sample in Joe and Clearwater lakes. Discrete trap samples from 1, 4, and 7 m were examined in Swan Lake.

TABLE 4. Model selected for correction of richness (y) for number of animals counted in individual samples (x). The range in average number of animals counted, by lake year, is also provided for each lake. Lakes are arranged in order of increasing distance from Sudbury.

Lake	Model selected	r^2	df	Range in no. of animals counted
Clearwater	Power	0.362	116	119–916
Swan	Power	0.202	41	367–711
Wavy	Power	0.251	51	387–610
Sans Chambre	Linear	0.136	32	232–409
Laundrie	Linear	0.487	51	243–628
Whitepine	Linear	0.179	83	227–385
Welcome	Power	0.234	51	507–628

increase in these lakes ranged from 0.16 to 0.43 species per year based on comparison of the earliest and latest years. No changes were detected in Welcome, the near-neutral reference lake, or Clearwater, Swan, and Whitepine lakes (Table 5; Fig. 2).

Corrected and uncorrected species richness values differed only for Swan and Whitepine lakes (Table 5). Decreases in species richness indicated by the uncorrected values in these lakes were attributable to high numbers of animals counted in the early years of study. This trend was no longer significant after data correction. These observations indicate that variation in effort of search was only a minor influence on patterns in the data set and that data adjustment was effective in removing spurious trends.

From three to nine additional species appeared in the more recent collections (Table 6) in Wavy, Laundrie, Sans Chambre, and Joe lakes based on comparisons between early and recent sampling years. One to five of these occurred in more than one sample and one to four were "common" (present in >50% of yearly samples). Between zero and five species collected in the early years became common in the most recent collections, while with one exception (*Orthocyclops modestus* in Sans Chambre lake), no species which was common in the early collections became rare (<20% occurrence) in the recent collections. Species recorded only in the early years were uniformly very scarce, essentially restricted to single occurrences. Their absence in recent collections may simply reflect sampling variability.

Species that were consistently present during the early and recent sampling periods for the Sudbury lakes are shown in Table 7. *Bosmina longirostris*, *Holopedium gibberum*, *Diaptomus minutus*, *Chydorus sphaericus*, *Mesocyclops edax*, and *Cyclops scutifer* were present during both sampling periods in over half the study lakes. All these are very common, widespread species in northern Ontario (Keller and Pitblado 1989) and with the possible exception of *C. scutifer* do not appear to be acid sensitive (Keller and Pitblado 1984). The absence of some of these species (*H. gibberum*, *D. minutus*, and *M. edax*) from the most heavily metal contaminated lakes (Table 2; Table 7) suggests an effect of trace metal toxicity.

The increases in average species richness observed in Wavy, Laundrie, Sans Chambre, and Joe lakes show that community recovery has occurred in some Sudbury area lakes following water quality improvements. However, only two of these four lakes, Laundrie and Joe, exhibited recent average species richness within the range observed in the Dorset reference lakes (Fig. 2). Even in these lakes, average richness did not fall within the 95% confidence limits for the average richness observed in the Dorset lakes ($\bar{x} \pm CI = 10.1 \pm 0.6$), indicating that the recovery of Sudbury lakes is not complete.

Factors Influencing Species Richness

Increases in average species richness may result from two different mechanisms, either invasion of new species or

TABLE 5. Comparison of ANOVA (GLM method (SAS 1987a)) results testing changes in corrected (C) and uncorrected (U) species richness against year using values for individual collections. Corrections standardizing species richness to counts of 250 animals were determined as described in the text. The average difference of uncorrected and corrected values for each lake and the change in the average species richness between early and recent sampling years are also indicated. Lakes are arranged in order of increasing distance from Sudbury.

Lake	Correction	F	p	Difference ($\bar{x}$) (U − C)	Change over time
Clearwater	U	1.11	ns[a]	0.14	0.10
	C	1.07	ns[a]		0.29
Swan	U	9.97	<0.01	0.04	−2.88
	C	1.49	ns[a]		−1.59
Wavy	U	13.63	<0.01	0.75	2.36
	C	10.54	<0.01		1.74
Joe	U	6.87	<0.01	—	2.85
	C	—	—	—	—
Sans Chambre	U	5.45	<0.01	0.34	1.57
	C	7.02	<0.01		1.55
Laundrie	U	18.43	<0.01	0.84	3.83
	C	10.63	<0.01		2.02
Whitepine	U	3.23	<0.01	0.26	−0.41
	C	0.75	ns[a]		−0.17
Welcome	U	1.18	ns[a]	1.08	1.31
	C	1.36	ns[a]		1.02

[a]No significant temporal change ($p > 0.05$).

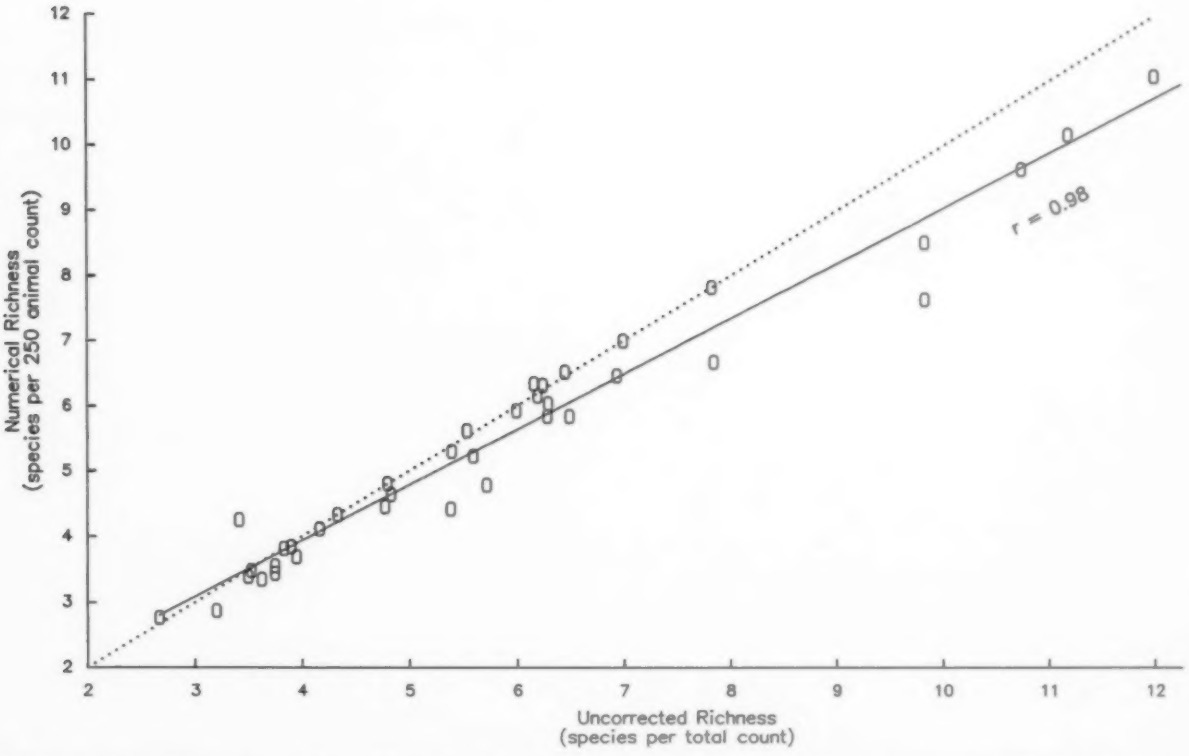

FIG. 1. Relationship between average species richness, by lake year, before and after adjustment of values to a counting effort of 250 animals. The dotted line represents a 1:1 relationship. The solid line is a regression model of corrected on uncorrected richness data.

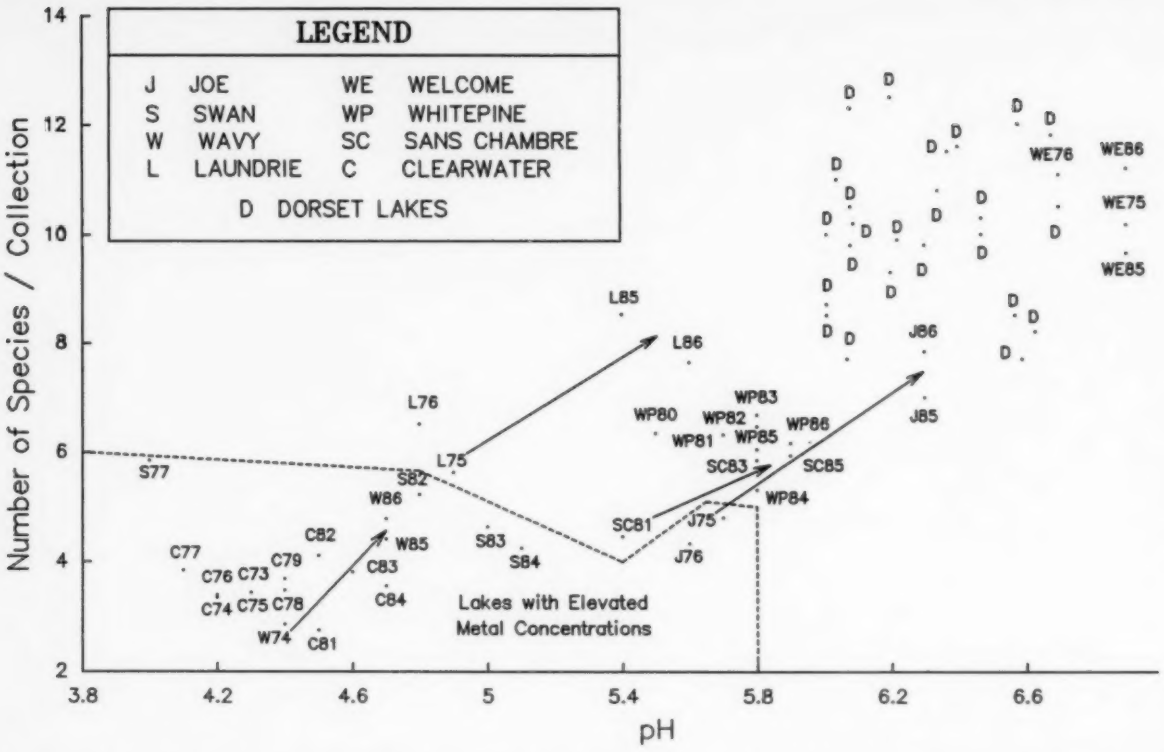

FIG. 2. Average number of crustacean plankton species per collection (adjusted to a constant counting effort of 250 animals, see Methods). The numbers in the lake identification codes indicate the year of sampling. Arrows indicate the approximate direction and extent of movement for lakes with significant (ANOVA, $p < 0.01$) changes in species richness. Lakes with elevated metal concentrations were defined as Cu > 5 $\mu g \cdot L^{-1}$ and Ni > 25 $\mu g \cdot L^{-1}$ (MOE 1984).

TABLE 6. Changes in the total number of crustacean plankton species and the occurrence of "new" (based on comparison of early and recent sampling periods) species in lakes showing increased average species richness. Where two adjacent sampling years were available at the beginning or end of the study, results for the two years were combined. Common species were present in >50% of the collections. Lakes are listed in order of increasing recent pH.

	Total no. of species		"New" species		
Lake	Present in earlier years	Present in latest years	"New"	Common	Present
Wavy	11	15	6	*Holopedium gibberum*	*Latona setifera* *Daphnia ambigua*[a] *Daphnia longiremis*[a] *Sida crystallina*[a] *Cyclops scutifer*[a]
Laundrie	18	19	5	*Eubosmina* sp.	*Daphnia longiremis*[a] *Daphnia retrocurva*[a] *Latona parviremis*[a] *Sida crystallina*[a]
Sans Chambre	8	9	3	*Daphnia pulex*	*Tropocyclops prasinus mexicanus* *Polyphemus pediculus*[a]
Joe	8	14	9	*Diaptomus oregonensis* *Epischura lacustris* *Tropocyclops p. mexicanus* *Daphnia pulex*	*Diaphanosoma* sp. *Daphnia galeata mendotae*[a] *Eubosmina* sp.[a] *Cyclops bicuspidatus thomasi*[a] *Eucyclops speratus*[a]

[a]Occurred on only one occasion.

TABLE 7. Species consistently present in the Sudbury study lakes during both early and recent sampling periods. Lakes are arranged in order of generally increasing recent pH.

Species	Clearwater	Wavy	Swan	Laundrie	Whitepine	Sans Chambre	Joe	Welcome
Alona sp.					×			
Bosmina longirostris	×	×	×	×	×	×	×	×
Ceriodaphnia sp.		×						×
Chydorus sphaericus	×	×	×	×	×			×
Daphnia ambigua				×	×			
Daphnia longiremis								×
Daphnia galeata mendotae					×			
Daphnia pulex		×		×	×			
Daphnia retrocurva								×
Diaphanosoma sp.		×		×				×
Eubosmina sp.			×					×
Holopedium gibberum				×	×	×	×	×
Latona setifera				×				×
Leptodora kindtii				×	×			×
Ophryoxus gracilis		×		×				
Polyphemus pediculus	×	×		×				×
Simocephalus serrulatus	×		×					
Sida crystallina								×
Diaptomus minutus		×		×	×	×	×	×
Diaptomus oregonensis								×
Epischura lacustris								×
Cyclops bicuspidatus thomasi					×	×		×
Cyclops scutifer				×	×	×	×	×
Cyclops vernalis	×							
Mesocyclops edax		×		×	×	×	×	×
Tropocyclops prasinus mexicanus				×	×			×

increased abundance of existing species through release from environmental stress. While we cannot demonstrate with certainty that species not collected were totally absent from these lakes, our observations do suggest that both the above mechanisms are important.

Only Joe Lake (recent pH 6.3) showed a substantial increase in the number of new "common" (present in more than 50% of the collections) species (*Epischura lacustris*, *Tropocyclops prasinus mexicanus*, *D. pulex*, and *Diaptomus oregonensis*) with time (Table 6), suggesting substantial recolonization. Synoptic survey results indicate that these species are widely distributed but acid sensitive (Sprules 1975; Keller and Pitblado 1984); thus, their importance as colonists is not surprising.

Only a single new "common" species was observed in recent collections from Sans Chambre (recent pH 5.9), Laundrie (pH 5.6), and Wavy (pH 4.7) lakes (*D. pulex*, *Eubosmina* sp., and *H. gibberum*, respectively). In Joe and Sans Chambre lakes, most (>90%) of the increased average species richness could be attributed to new species, primarily the single species *D. pulex*, in Sans Chambre Lake. In Wavy and Laundrie lakes, new species could account for only 57 and 46%, respectively, of the increased average species richness, suggesting that expansion of existing species was also very important.

The extent of improvement in species richness in the study lakes is likely influenced by many factors, including ambient chemistry, presence of recolonization sources, and biological interactions.

Except for Welcome Lake, the nonacidic reference lake, only one of the Sudbury study lakes, Joe Lake, exhibited a recent pH of >6.0 and only two others (Whitepine and Sans Chambre) had pH ≥ 5.7. Some acid-sensitive zooplankton species including *Daphnia galeata mendotae* and *E. lacustris* decline at pH near 6.0 (Keller and Pitblado 1984; Yan et al. 1988; Keller et al. 1990). These acid-sensitive species occurred only in Sudbury lakes of pH ≥ 5.7, although they typically have a very ubiquitous distribution (88 and 71% of the near-neutral Dorset lakes for *D. g. mendotae* and *E. lacustris*, respectively). The recolonization of Wavy Lake by *H. gibberum* likely reflects the temporal decreases in trace metal concentrations in the lake (Table 2), since this species is acid tolerant, but metal sensitive (Lawrence and Holoka 1987). While significant increases in overall species richness have occurred in some Sudbury lakes of low present pH (e.g. Wavy, pH 4.7), current levels of acidity, compounded by elevated metal levels in the lakes closest to Sudbury, undoubtedly continue to constrain the development of normal, diverse crustacean faunas in many Sudbury area lakes.

While they may greatly affect recovery rates, external sources for colonization are likely not ultimately limiting for our study lakes. Near-neutral lakes occur in proximity (<5 km; usually <1 km) to all these lakes, and natural agents for colonization such as aquatic Hemiptera (Schlichting and Sides 1969) are common. Dispersal problems may be more pronounced in other areas within the Sudbury region that contain pockets of uniformly acid lakes; however, it is important to note that in these lakes and others, the sediments may constitute an important colonization source. Carvalho and Wolf (1989) and De Stasio (1989) have shown that viable resting eggs of several zooplankton species can persist for long periods in lake sediments. It is interesting that in Wavy and Laundrie lakes, crustacean species normally considered most typical of the littoral zone (Ward and Whipple 1966), *Sida crystallina* and *Latona* sp., appeared in the most recent open-water collections (Table 6). This suggests that they may have expanded from littoral refugia following water quality improvements.

The lack of change in community richness in Whitepine, Clearwater, and Swan lakes may be due to several influences. The apparent lack of response in Whitepine Lake may simply

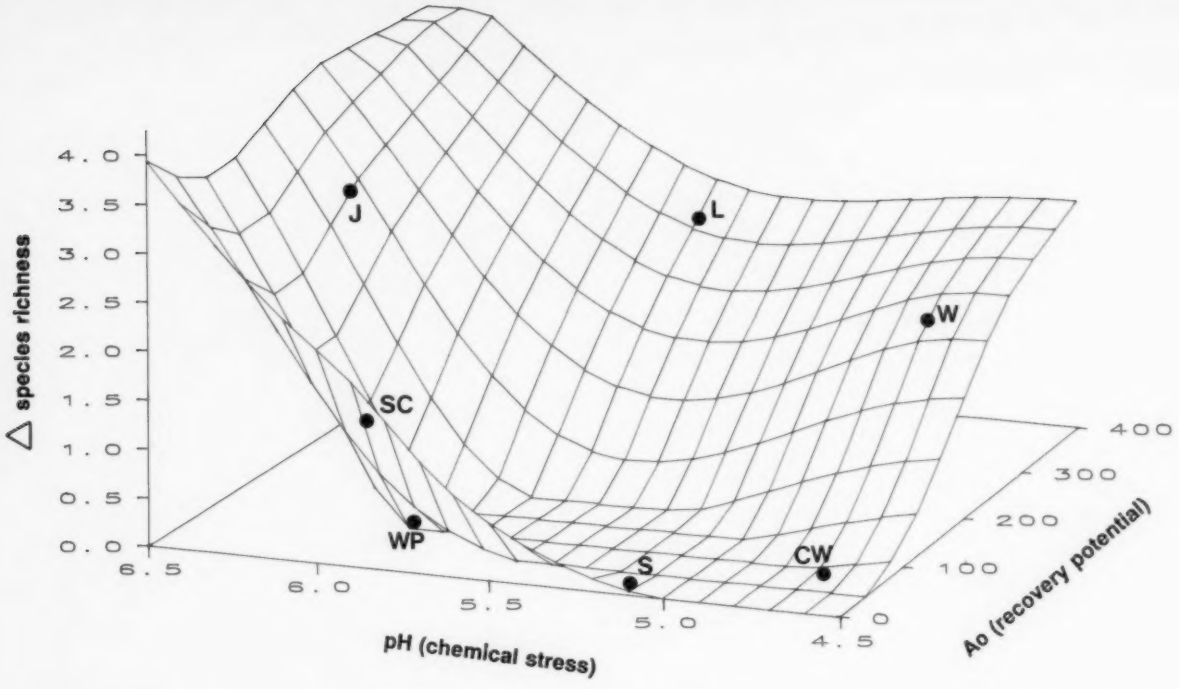

FIG. 3. Three-dimensional response surface of changes in species richness in the study lakes as related to recent pH and lake area (ha). Lake identification codes are given in Fig. 2. Change in species richness is the difference in the average number of species per collection between early and recent sampling periods. The rectangular grid of interpolated values was produced using SAS (1987b).

reflect the modest pH shift (5.5–5.9) and comparatively short study period (7 yr) for this lake. It is noteworthy that although Whitepine Lake did not exhibit increased average species richness, at least one new acid-sensitive species (*E. lacustris*) has recolonized the lake (Gunn and Keller 1990). Despite substantial water quality improvements in Clearwater and Swan lakes, highly acidic, metal-contaminated conditions persist, undoubtedly causing or contributing to the continuing community impoverishment, particularly in Clearwater Lake which has the most extreme current chemistry (Table 2) among the study lakes. While high acidity and metal contamination are certainly involved in suppressing species richness in Swan Lake, biological influences may also be important.

Yan et al. (1991) have shown that the very high abundance of *Chaoborus* in Swan Lake exerts a major control on the abundance and composition of the lake's zooplankton community. Intense invertebrate predation related to the absence of fish may, therefore, restrict the invasion of some new species to Swan Lake, consistent with the findings of Von Ende and Dempsey (1981) and Nyberg (1984). *Chaoborus* data for our other lakes are very limited, but the lack of recovery observed in Clearwater and Whitepine lakes is not attributable to *Chaoborus* predation, since both these lakes have very low *Chaoborus* populations (autumnal abundance <2 animals·m^{-3}; Yan et al. 1985). In contrast, crustacean species richness increased in San Chambre Lake despite a large *Chaoborus* population (204 animals·m^{-3}; Yan et al. 1985).

With the exception of Swan and Clearwater lakes, all of the study lakes have continuously supported fish populations, although dramatic changes in community composition have occurred in some cases. The observed shifts in fish species abundances, typically from forage species such as *Perca flavescens* to game species such as *Salvelinus namaycush, Salvelinus fontinalis*, and *Micropterus dolomieui* (J. M. Gunn, unpubl. data), involve species that are all likely *Chaoborus* predators. Since food availability for early instar *Chaoborus* larvae (Neill and Peacock 1980), not the presence of fish (Yan et al. 1985), appears to regulate *Chaoborus* abundance in oligotrophic systems, *Chaoborus* abundances are likely low in our other study lakes because of food limitation. Thus, while intense invertebrate predation may be an important factor affecting recolonization in small, relatively nutrient-rich, fishless lakes such as Swan Lake, this mechanism is not likely of general importance in our study lakes.

Changes in direct predation on crustacean plankton by fish may also potentially favour species recolonization, through changes from dominance by small planktivorous forage species to larger gamefish species that primarily utilize plankton during only their earlier life stages. For example, in Joe Lake, there was a shift from *Etheostoma exile* to a reintroduced population of *S. fontinalis* (J. M. Gunn, unpubl. data). Fish predation can certainly alter the abundance and size structure of crustacean zooplankton communities (e.g. Raess and Maly 1986; Mills et al. 1987; Post and McQueen 1987); however, effects on zooplankton diversity are not clear.

Prediction of Recovery in Species Richness

Obviously, many factors including initial degrees of impact, extent of water quality improvements, available recolonization

time, and the dispersal capabilities of potential colonizers may greatly influence the rate and extent of zooplankton community recovery accompanying water quality changes.

With our limited lake set, it is difficult to quantify these factors, but collectively our observations support a very simple recovery model (Fig. 3) that predicts the greatest increases in species richness among larger, deeper lakes with abundant inflows (reflecting the overall potential for both internal and external recolonization sources) and substantial water quality improvements, and the least increases in lakes with fewer colonization sources or lakes with minimal water quality changes. Lake pH is negatively related to trace metal concentrations; thus, the pH axis represents a gradient of general chemical stress. Since lake area is positively related to lake depth and the number of inflowing waterbodies (as determined from 1 : 50 000 topographic maps), the area axis is interpreted as the general internal and external "recovery potential" encompassing information on number of colonization opportunities and habitat diversity. This preliminary, generalized model should prove helpful as a framework for further study, although additional data will be required to determine its general usefulness and applicability.

Given suitable ambient water quality, recolonization by common zooplankton species ultimately may be most dependent on time available for dispersal. Because of varying degrees of water quality improvements, the temporally discontinuous nature of most of our data base, and other lake-specific complicating factors, extrapolation of our average recovery rates to other systems would be tenuous. Further, because the exact preimpact composition of zooplankton assemblages in these lakes is not known, we cannot demonstrate a return to historical conditions, only movement toward communities typical of the region. However, it is clear that water quality improvements do lead to substantial recovery of crustacean zooplankton species richness in less than a decade, even in lakes that were very stressed. These results demonstrate that reductions in acid-forming emissions can rapidly lead to biological as well as chemical improvements in aquatic ecosystems.

Acknowledgements

We thank J. Carbone, P. Gale, R. Girard, and R. Griffiths for technical assistance and W. Geiling, L. Sardella, and B. Cave for zooplankton identifications. P. Dillon, J. Gunn, J. Carbone, D. Malley, P. Chang, and N. Watson provided constructive reviews of this paper. Funding was partially provided under the Acidic Precipitation in Ontario Study (APIOS) of the Ontario Ministry of the Environment.

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Physicochemical Patterns within a Hyporheic Zone of a Northern Michigan River, with Comments on Surface Water Patterns[1]

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Interstitial temperature and chemistry were examined longitudinally and with depth in a hyporheic zone beneath a riffle–pool sequence of a third-order, sand-bottom river in northern Michigan (USA). Longitudinal and depth patterns were compared with surface and groundwater chemistries at the site and with surface chemistry over a 10-km length of the river. Interstitial water was more characteristic of surface water at the upstream end of the hyporheic zone and of groundwater at the downstream end. Hyporheic longitudinal and depth patterns occurred for temperature, chloride, silica, soluble reactive phosphorus, and winter dissolved oxygen. Dissolved organic carbon decreased with depth, but not with longitudinal distance downstream within the hyporheic zone. Hyporheic nitrate and ammonium patterns were variable, but concentrations generally increased downstream. Hyporheic specific conductance and alkalinity decreased with distance downstream, but not with depth. Gradients for temperature, chloride, silica, nitrate, and phosphate over the 10-km surface water reach were similar to those observed within the single hyporheic zone. Hyporheic chemistry patterns were consistent with models of underflow through porous substrata implying groundwater/surface water interaction. Importance of the hyporheic zone should not be overlooked in solute transport or stream nutrient budgets.

On a examiné, longitudinalement et en fonction de la profondeur, la chimie et la température intersticielle d'une zone hyporhéique formant le fond d'une alternance de seuils et de fosses, dans une rivière sablonneuse de troisième ordre située dansa le nord du Michigan (É.-U.) Les caractéristiques relevées en profondeur et longitudinalement ont ensuite été comparées avec la chimie des eaux de surface et souterraines du site et avec la chimie de surface d'un tronçon de 10 km de la rivière. Les eaux intersticielles ressemblaient davantage aux eaux de surface dans la partie amont de la zone hyporhéique et aux eaux souterraines dans la partie aval. Les caractéristiques de la zone hyporhéique se retrouvaient sur le plan longitudinal et en profondeur pour la température, le chlorure, le silice, le phosphore réactif soluble et l'oxygène dissout en hiver. Le taux de carbon organique dissout diminuait avec la profondeur mais non avec la distance longitudinale vers l'aval au sein de la zone hyporhéique. Le nitrate et l'ammonium étaient répartis de façon variable au sein de la zone hyporhéique mais, de manière générale, les concentrations augmentaient à mesure que l'on descendait vers l'aval. L'alcalinité et la conductivité spécifique de la zone hyporhéique diminuaient avec l'éloignement vers l'aval, mais non en fonction de la profondeur. Les gradients de température, de chlorure, de silice, de nitrate et de phosphate sur les 10 km d'eau de surface étaient similaire à ce que l'on a observé au sein de la seule zone hyporhéique. Les caractéristiques chimiques de celle-ci étaient conformes aux modèles d'inféro-flux dans le substrat poreux, ce qui implique une interaction entre les eaux souterraines et de surface. L'importance de la zone hyporhéique ne devrait pas être négligée dans les bilans de transport des solutés ou des nutriments de cours d'eau.

Received December 12, 1989
Accepted April 8, 1991
(JA402)

Reçu le 12 décembre 1989
Accepté le 8 avril 1991

Among the least understood and studied components of stream ecosystems are the physicochemical and biological patterns and processes within hyporheic zones of streambeds (Hynes 1983), particularly where surface water and groundwater interact. Groundwater may contribute directly to surface water discharge through springs and seeps depending on local geology and hydrology (Freeze and Cherry 1979). Groundwater also may percolate through the hyporheic zone mixing with interstitial water in the bed sediments (Williams 1984).

Surface water has been shown to establish regular and predictable interstitial underflow patterns beneath wave-form riffles dependent on such factors as streambed permeability, surface water discharge, hydraulic gradient, and longitudinal bed-surface contour (Vaux 1968; Thibodeaux and Boyle 1987). In the basic hyporheic underflow model, surface water downwells into the bed at the upstream end of a riffle, travels for some distance beneath the riffle, and then upwells and exits at the downstream end (Fig. 1). Water exiting the bed theoretically should reflect biogeochemical processes which have occurred within the bed. If underflow also implies mixing with

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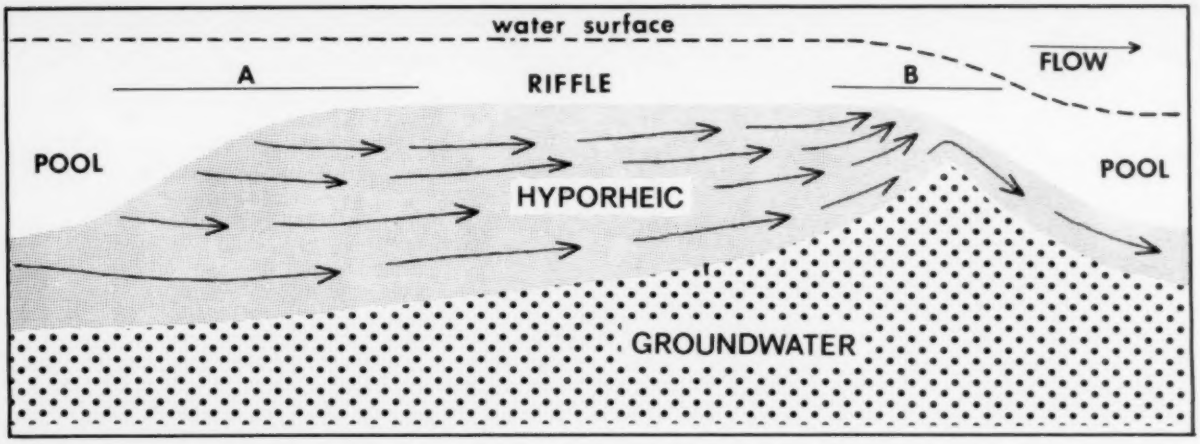

FIG. 1. Conceptual diagram of surface water movement pattern (underflow) through the hyporheic zone of a streambed pool–riffle–pool sequence and the relation to groundwater distribution. Line A represents the region of surface water infiltration (downwelling) and line B represents the region of expected underflow return (upwelling) to the surface (after Vaux 1968; Thibodeaux and Boyle 1987; White et al. 1987).

groundwater in predictable areas within the streambed, then longitudinal chemical and biological patterns resulting from water mass interactions also might be predictable.

Past studies (reviewed in Williams 1984, 1989) have demonstrated chemical and biological gradients vertically with depth into bed sediments and laterally with distance beneath stream banks. With the exception of temperature (White et al. 1987; Hendricks and White 1988; White 1990), longitudinal gradients, particularly in relation to underflow patterns, have not been examined.

The Maple River, a third-order river in northern Michigan, USA, has provided some ideal conditions for examining hyporheic patterns, especially surface water and groundwater mixing. The river has only two major sources of water, the Douglas Lake outflow and groundwater. Douglas Lake outflow water temperature and quality are dependent on limnological cycles within the lake. Groundwater temperature and quality are more constant and distinctly different from Douglas Lake water in a number of chemical characteristics. The primary objective of this study, therefore, was to describe and map hyporheic physicochemical patterns beneath a riffle–pool segment. Hyporheic temperatures had previously suggested regular mixing patterns of the two water masses within the hyporheic zone (White et al. 1987). Based on the temperature studies, we postulated that surface water and groundwater interactions should result in concentrations of substances dependent on location along longitudinal underflow paths, i.e. with depth into the bed and with distance downstream from the head of a riffle. Following initial hyporheic studies, we examined a longer reach of the river to determine if the cumulative discharge of a number of hyporheic zones might be reflected in surface water quality.

Materials and Methods

Study Stream

The East Branch of the Maple River is located almost entirely on University of Michigan Biological Station property near Pellston, Michigan (Fig. 2). Hydrologic, geologic, and biotic features of the river have been described previously (White et al. 1987; Fortner and White 1988; Hendricks and White 1988; White 1990). The study reach is approximately 10 km in length beginning at the Douglas Lake outlet (Station 1) and ending at Station 7. Much of the stream between Stations 1 and 6 flows through second-growth aspen (*Populus tremuloides* Mich., *P. grandidentata* Michx.), white birch (*Betula papyrifera* Marsh), and red maple (*Acer rubrum* L.). Occasional beaver meadows occur between Stations 1 and 2 with no active impoundments. Van Creek, the only tributary, is an intermittent stream usually dry except during spring snowmelt. Active beaver impoundments were present between Stations 6 and 7 in 1988. The number of dams increased between the 1988 and 1989 sampling periods with additional dams appearing between Stations 4 and 5. The Maple River flows over an unconfined aquifer with sandy, glacial till substrata up to 100 m deep in the study reach.

Hyporheic Sampling

The hyporheic site is a riffle–pool segment 10 m in length and 7–8 m wide located at Station 2 (corresponding to Site 3 in White et al. 1987). The riffle surface is overlain by a shallow layer of small to medium gravel (3–5 cm in diameter). The substratum >10 cm deep beneath the riffle surface is composed of approximately 63% medium sand and 22% fine sand to depths of 50 cm. The site has been permanently gridded (1-m^2 quadrats, White et al. 1987) and is maintained as a permanent research site by the Biological Station.

The main underflow gradient had been shown to be in the downstream direction along the midstream transect of nine quadrats (White et al. 1987) which was confirmed by earlier dye studies (S. P. Hendricks, unpubl. data). The first and last quadrats are in the upstream and downstream pools, respectively, and the remaining quadrats cover the length of the riffle.

Three independent water samples were collected at the sediment–water interface and at 10-cm depth intervals down to 50 cm deep into the substratum in each quadrat on all sampling dates. Water was collected with a well-point sampler (2-cm diameter) fitted with Tygon tubing (4.7-mm inside diameter) atached to a hand-held vacuum pump and an Erhlenmeyer flask. The sampler was driven to the selected depth and allowed to stabilize for 2–3 min before pumping. Approximately 100 mL

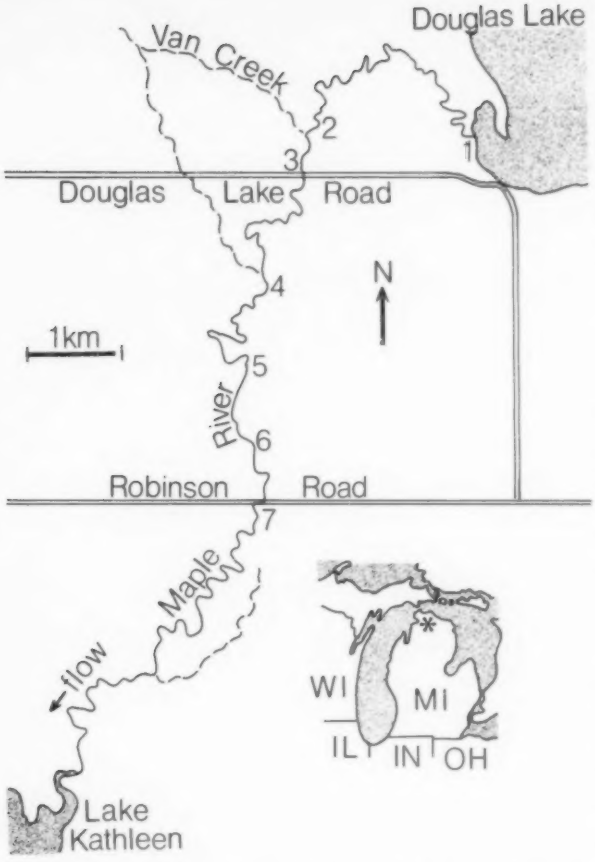

FIG. 2. Map of station locations on the East Branch of the Maple River near the University of Michigan Biological Station. Inset shows the approximate location of the study area in northern Michigan (USA).

of interstitial water was slowly pumped through the sampling apparatus as a rinse. A subsequent sample of 150 mL was collected and split into 100 mL for nutrient and 50 mL for dissolved organic carbon (DOC) analyses. Nutrient samples were placed in sterile Nasco Whirl-pak bags, iced in a cooler, and transported immediately to the laboratory. DOC samples were placed into sterile, acid-rinsed glass bottles and preserved to pH 2 with phosphoric acid (APHA et al. 1989). Bed temperatures were recorded at 10-cm depth intervals to 50 cm deep using a 95-cm temperature probe and thermistor (YSI series 400). Temperature data were used not only to map patterns but also to verify that the appropriate water masses had been sampled.

Hyporheic measurements were made during base-flow periods in August 1987–89 and February 1987 when greatest temperature differences occurred between Douglas Lake outflow water and groundwater and when base-flow was expected to produce the most marked chemical differences between the two water masses. Unless indicated, data presented are from August 1987 and are annotated for exceptions in other years. February patterns are presented where particular variables were distinctly different from August patterns.

Groundwater and Whole-Reach Surface Water Sampling

Groundwater samples were collected from a lateral well placed about 3 m from the stream at Station 2. The well was pumped dry two to three times and allowed to refill prior to nutrient and DOC sample collection. Surface water was collected in triplicate at seven stations (Fig. 2) concurrent with hyporheic sampling during August 1988 and 1989 to examine whole-reach patterns of inorganic nutrients and DOC. Stream velocity and discharge were measured at each station at 1-m intervals from bank to bank with a Teledyne pygmy-Gurley meter.

Chemical Methods

Temperature, dissolved oxygen (DO), pH, redox potential (E_h), total dissolved solids, specific conductance, and alkalinity were determined in the field. DO was measured with a YSI dissolved oxygen meter (model 57) or by micro-Winkler titration (APHA et al. 1989). pH was measured with a Cole-Parmer pH probe and meter. E_h and total dissolved solids were determined using field meters (Hanna Instruments). Specific conductance was measured with a YSI specific conductance meter (model 33). Alkalinity (milligrams $CaCO_3$ per litre) was determined by titration with 0.02 N H_2SO_4 to pH 4.5 (APHA et al. 1989).

Nutrient samples were filtered through Millipore H/A membrane filters (0.45-μm pore size), transferred to acid-rinsed polyethylene bottles, and stored frozen until analysis. Filters were frozen for chlorophyll *a* and later analyzed by a fluorometer (Turner Designs). Samples were thawed overnight prior to analysis for soluble reactive phosphorus (SRP), nitrate + nitrite (henceforth referred to as nitrate), ammonium, and chloride. Silica (SiO_2) was determined after samples were thawed for 48 h. Nutrients were analyzed by either Technicon II or Lachat autoanalyzers following the methods of APHA et al. (1989) and others (Table 1). DOC samples were filtered through precombusted Gelman A/E glass fiber filters (0.45-μm pore size). DOC was determined by the ampoule-persulfate digestion method (Menzel and Vacarro 1964, with modification of McDowell et al. 1987) using Oceanography International total carbon analyzers (Model 524 or 700) equipped with infrared CO_2 detectors. E_h, total dissolved solids, chlorophyll *a*, and ammonium were measured in 1989 only.

Isopleth Plots and Statistical Analyses

Streambed isopleths were determined and plotted using software and contour plotting programs of SYGRAPH and SYSTAT (Wilkinson 1988, 1989). In this paper, we considered the datum to be the streambed and not the stream water surface. This was the simplest approach and visually easier to interpret. Further, the streambed was the stable reference point, while the water surface fluctuated among sampling dates. Our primary interests were in overall physicochemical patterns more than absolute values. Thus, we have not included thermal conduction or porosity measurements, and we have not quantified seepage flux rates.

Hyporheic data were analyzed by depth into the bed with the substratum – surface water interface being zero depth and by distance which referred to the downstream axis beneath the riffle. Most hyporheic, surface water, and groundwater data were normally distributed following log transformation. Hyporheic temperature, general chemical data, nutrients, and DOC data

TABLE 1. Methods used in water chemistry analyses.

Nutrient	Method	Reference	Detection limit (μg/L)
PO_4-P	Molybdenum blue	Murphy and Riley 1962	1.5
NO_3-N	Cadmium reduction	Armstrong et al. 1967	10–15
NH_4-N	Phenolypochlorite	Solorzono 1969	50
SiO_2	Molybdenum blue	Strickland and Parsons 1968	20
Cl	Ferricyanide	O'Brien 1962	100
DOC	Persulfate digestion	Menzel and Vacarro 1964	200

TABLE 2. Means (SD) or ranges of 15 physicochemical variables for surface water and groundwater during mid-August (1986–89) base-flow conditions at Station 2 on the Maple River (East Branch), Emmet Co., Michigan, USA. The *t*-statistic for means is significantly different ($p < 0.05$) for all variables except discharge.

Variable	Surface water	Groundwater
Discharge (m^3/s)	0.19 (0.04)	—
Temperature (°C)	26 (3)	8.5 (1)
DO (mg/L)	6.5–8.0	1.0–2.0
E_h (mV)	229 (2)	45 (3)
Alkalinity (mg $CaCO_3$/L)	130 (5)	201 (2)
pH	7.5–8.5	6.5–7.0
Total dissolved solids (μg/L)	144 (10)	210 (8)
Specific conductance (μS/cm)	174 (4)	300 (5)
Phosphate (SRP) (μg/L)	1.12 (1.04)	2.41 (0.30)
Nitrate (NO_2 + NO_3) (μg/L)	28.9 (15.2)	14.8 (3.2)
Ammonium (NH_4) (μg/L)	67.3 (41.3)	176.4 (16.1)
Silica (SiO_2) (mg/L)	3.1 (0.7)	7.3 (3.0)
Chloride (mg/L)	3.8 (0.2)	1.1 (0.2)
DOC (mg/L)	12.4 (2.4)	2.5 (1.5)
Total chlorophyll *a* (μg/L)	4.0 (0.7)	1.3 (0.4)

were analyzed by two-way analysis of variance (ANOVA) programs of SYSTAT (Wilkinson 1989). Scheffe's all-pairs comparison test was used for examination of significant differences between depths and downstream locations within the hyporheic zone. Hyporheic data not normalized by transformations (nitrate, ammonium, and DOC) were analyzed by nonparametric Friedman's two-way ANOVA. Whole-reach surface water data were analyzed by linear regression, while means of surface water and groundwater variables were compared with *t*-tests. Levels of significance were set at $\alpha = 0.05$ for all statistical tests.

Results and Discussion

Longitudinal Hyporheic Patterns

Decreases in temperature with depth into the substratum during summer have been assumed to be related to the presence of groundwater (Sheridan 1962; Hartman and Leahy 1983; Shepherd et al. 1986; Williams 1984, 1989). Temperature was used initially to differentiate surface water from groundwater within this and other hyporheic study sites in the Maple River (White et al. 1987; White 1990), and additional data have shown that patterns generally remain consistent from season to season and year to year. Summer surface water temperatures at Station 2 were as high as 26–27°C, while groundwater remained at 8–9°C (Table 2). Hyporheic isopleths in a typical August pattern (1988) indicated that distinctly warmer surface water downwelled into the substrata to depths of 50 cm or more at the upstream head of the riffle, while cooler water upwelled nearer the substratum – surface water interface beneath the downstream end of the riffle and the pool (Fig. 3; $p < 0.01$). Hyporheic temperature patterns were reversed in February when groundwater was warmer than surface water (also see Sheridan 1962).

Naturally occurring chloride has rarely been measured in stream sediments, but it has been used as a tracer for underflow through gravel and sand substrata because of its conservative and nonadsorptive behavior (Jackman et al. 1984; Bencala 1984; Triska et al. 1989, 1990) and also has been used to track groundwater through aquifers (Lee and Cherry 1978). August Station 2 surface water chloride concentration was about 4 mg/L and consistently around 1 mg/L in groundwater (Table 2). Hyporheic chloride concentrations were always less than surface water concentrations and decreased with depth into the bed (Fig. 3) approaching groundwater concentrations at 40- to 50-cm depths. The decrease into the bed suggested groundwater dilution of chloride with increased groundwater discharge upward through the hyporheic zone at the downstream end.

Ford and Naiman (1989) found a fivefold difference between silica concentrations in groundwater and surface water and suggested that groundwater was the primary source of surface water silica (silicon) in boreal streams. Station 2 surface water silica concentration was about 3 mg/L in August 1988 (Table 2) but had been as high as 6 mg/L during other years. Groundwater concentrations were 7–9 mg/L in 1988 and as high as 12 mg/L during other years. Hyporheic silica increased with increasing depth and distance and approached groundwater concentrations at the downstream end of the zone at the 40- to 50-cm depths (Fig. 3; $p < 0.05$).

Nitrogen concentrations (nitrate, ammonium) have been shown to be generally higher in the hyporheic zone than in

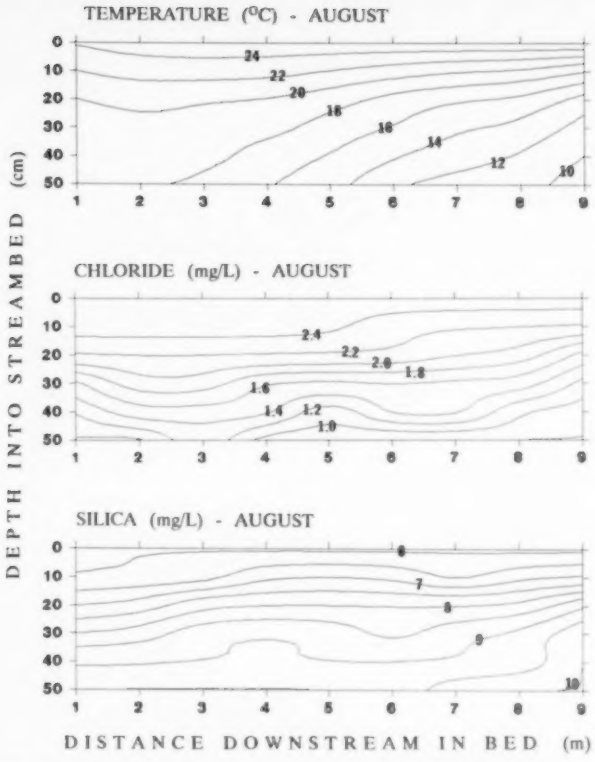

FIG. 3. Hyporheic isopleths of a typical August (1987–89) temperature pattern and chloride and silica patterns along the central longitudinal transect beneath the riffle at Station 2. Metre 1 is at the upstream end of the hyporheic zone and metre 9 is the downstream end. Datum 0 is the streambed surface. $n = 3$ for each depth sampled at each site beneath the riffle.

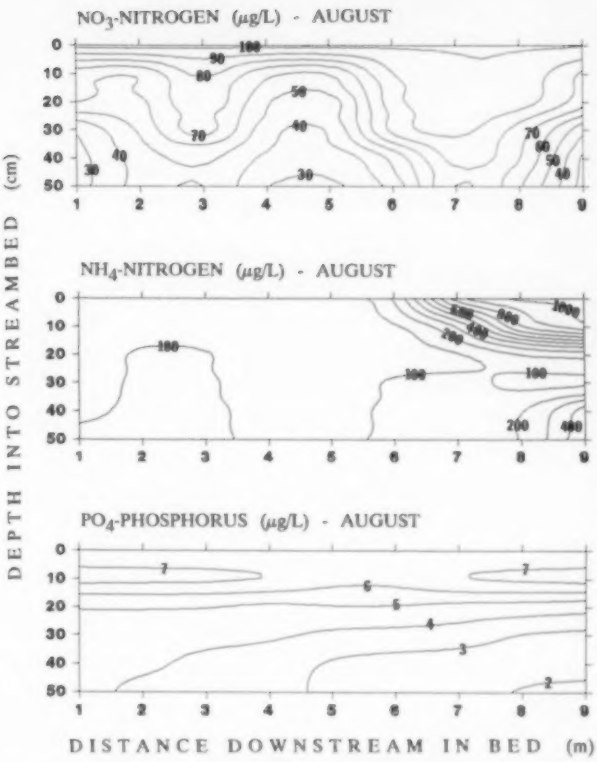
NO3-NITROGEN (μg/L) - AUGUST
NH4-NITROGEN (μg/L) - AUGUST
PO4-PHOSPHORUS (μg/L) - AUGUST
DEPTH INTO STREAMBED (cm)
DISTANCE DOWNSTREAM IN BED (m)

FIG. 4. Hyporheic isopleths of typical nitrate, ammonium, and SRP patterns along the central longitudinal transect beneath the riffle at Station 2 during August base-flow conditions (1988 and 1989). $n = 3$ for each depth sampled at each site beneath the riffle. See Fig. 3 for description of axes.

surface waters, particularly in hardwater streams (Williams 1984, 1989; Triska et al. 1990; Valett et al. 1990) and to decrease with depth into the bed (Schwoerbel 1961; Williams 1989). Surface water nitrate (28.9 μg/L) was twice that of groundwater (14.8 μg/L) at Station 2 in August 1987. Hyporheic nitrate was patchy but always enriched (Fig. 4) with concentrations three times that of surface water and five to six times that of groundwater. Although hyporheic nitrate decreased with depth, there was an overall increase with distance downstream beneath the riffle (Fig. 4). Thus, both depth and distance were significant factors ($p < 0.001$) explaining nitrate distribution patterns.

Ammonium was measured only in August 1989. Surface water ammonium at Station 2 was extremely variable, averaging 67 μg/L (Table 2). Groundwater ammonium was higher and less variable, averaging 176 μg/L. Hyporheic ammonium was patchy but increased with both depth and distance downstream beneath the riffle (Fig. 4; $p < 0.001$). The highest ammonium concentration (>1000 μg/L) occurred at the 10-cm depth at the downstream end of the riffle. This location in August 1989 also had an unusually low nitrate concentration (5.4 μg/L), the lowest redox potential (−113 mV), and the lowest dissolved oxygen level (2.2 mg/L).

Phosphate is often substantially higher in the first 10–20 cm of a hyporheic zone than in surface waters (Schwoerbel 1961; Valett et al. 1990) or deeper in the bed (Williams 1989). SRP in surface water (1 μg/L) and groundwater (2 μg/L) at Station 2 were significantly different ($p < 0.01$) (Table 2). Hyporheic SRP at 10 cm deep was always three to four times the concentration of surface water or groundwater SRP. Hyporheic SRP decreased with both depth beyond 10 cm and with distance beneath the riffle during base-flow conditions (Fig. 4; $p < 0.001$). As with ammonium in August 1989, high concentrations of SRP were found at the 10- and 20-cm depths at the end of the riffle where reducing conditions occurred.

The amount and distribution of hyporheic DO is highly variable among stream systems but in general has been suggested as strong evidence for surface water – interstitial exchange (Sheridan 1962; Godbout and Hynes 1982; Grimm and Fisher 1984). Whitman and Clark (1982), Williams (1989), and Giberson and Hall (1988) had reported decreases in concentrations with depth into the bed. Surface water DO at Station 2 ranged from 6.5 to 9.0 mg/L during August base-flow conditions and up to 12 mg/L in February. Groundwater DO remained fairly consistent year-round at about 2 mg/L. Hyporheic DO gradients typically were less distinct in August than in Feburary (Fig. 5), displaying a patchy distribution particularly beneath the downstream end of the zone. DO patterns in February were more regular and conform to gradients found by others (Sheridan 1962; Valett et al. 1990). Concentrations remained near that of surface water down to depths of 50 cm in downwelling regions at the upstream head

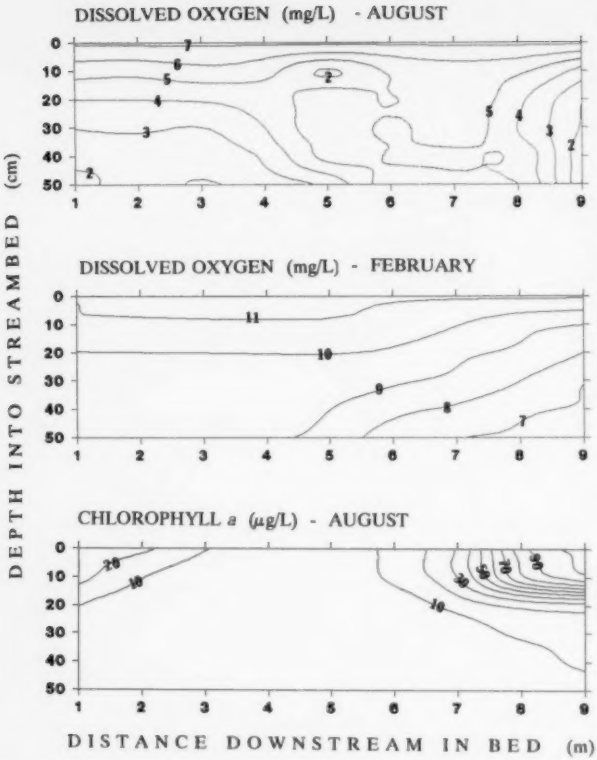
DISSOLVED OXYGEN (mg/L) - AUGUST
DISSOLVED OXYGEN (mg/L) - FEBRUARY
CHLOROPHYLL a (μg/L) - AUGUST
DEPTH INTO STREAMBED (cm)
DISTANCE DOWNSTREAM IN BED (m)

FIG. 5. Hyporheic isopleths of typical August (1987) and February (1987) DO and chlorophyll *a* (1989) patterns along the central longitudinal transect beneath the riffle at Station 2. $n = 3$ for each depth sampled at each site beneath the riffle. See Fig. 3 for description of axes.

of the riffle. DO decreased ($p < 0.001$) with distance beneath the riffle, with lowest concentrations occurring in upwelling regions.

Surface water infiltration during August base-flow conditions may have been insufficient to renew DO beneath the downstream end of the zone. The region of high ammonium, SRP, and low nitrate concentrations at the 10- and 20-cm depths possibly resulted from the breakdown of the oxygen gradient during higher summer temperatures and higher sediment oxygen demand. Similar relationships have been described by Sheridan (1962), Grimm and Fisher (1984), and Ford and Naiman (1989). Anoxia was never observed above 50-cm depths in either February or August. Redox potential (E_h) was measured in three quadrats in August 1989: upstream end of the riffle, midriffle, and downstream end. Hyporheic E_h was almost as patchy at the downstream end as was DO. Lowest E_h was found at the 10- and 20-cm depths at the downstream end of the riffle, -113 and -34 mV, respectively. Average E_h at the upstream end (144 mV) was not significantly different from surface water (136 mV). Surprisingly, correlations between E_h and DO actually improved along the downstream gradient: upstream end, $r = 0.14$; midriffle, $r = 0.79$; and downstream end, $r = 0.99$. Smaller anaerobic areas may have existed locally or at particle surfaces throughout the hyporheic zone that were undetected by our methods.

Chlorophyll *a* rarely has been measured in deeper sediments; however, Lush and Hynes (1978a) associated high pigment concentrations with high particle concentrations in surface sediments. Chlorophyll *a* at Station 2 ranged from 3.5 μg/L in surface water to about 1.0 μg/L in groundwater (Table 2). Chlorophyll *a* significantly decreased ($p < 0.001$) with depth in the substratum and increased with distance beneath the riffle (Fig. 5). Highest concentrations (120 μg/L) occurred shallow (10-cm depth) in the downstream end of the hyporheic zone. Lowest concentrations were usually found at 50-cm depths. A preliminary study of phaeophytin content in a subset of samples indicated that it constituted approximately 50% of the pigment measured as chlorophyll *a* in surface water, 55% in shallow hyporheic water (10- to 30-cm depths), and 70% in deeper hyporheic water (40- to 50-cm depths). Phaeophytin also constituted more than 70% of the total pigments measured as chlorophyll *a* in groundwater. These data suggest different origins for shallow and deeper hyporheic pigments.

As with phosphate and nitrate, hyporheic DOC is often more concentrated at the 10- to 20-cm depths than in surface waters or deeper in the bed (Crocker and Meyer 1987; Rutherford and Hynes 1987). Groundwater does not seem to be a significant DOC source to stream systems except in some mountain areas with very low surface water DOC concentrations (Wallis et al. 1981). Surface water DOC was about 12 mg/L during August 1986 and 1987 and 8 mg/L during February 1987 at Station 2. Groundwater DOC was relatively low and constant at about 4 mg/L. Hyporheic DOC was always enriched relative to both surface and groundwater concentrations ($p < 0.01$). Composited DOC data from August 1986 and 1987 showed decreases with depth into the substratum ($p < 0.01$) but not with distance downstream (Fig. 6).

Hyporheic alkalinity has been shown to be quite variable but generally higher than surface water alkalinity, particularly in hardwater systems (Williams 1984). Surface water alkalinity ($CaCO_3$) at Station 2 during August was 130 mg/L, and groundwater alkalinity was 200 mg/L (Table 2). There was no gradient for alkalinity with depth into the substratum. Composited data for all August and February sampling periods showed that alkalinity decreased from 155 mg/L at the upstream end to 128 mg/L at the downstream end (Fig. 7; $p < 0.001$).

Several studies have noted that the pH of hyporheic water is often up to 1 unit lower than surface water pH (Schwoerbel 1961; Husmann 1971; Williams and Hynes 1974; Williams 1984, 1989). Station 2 surface water pH was always significantly higher (0.5–1 pH unit, $p < 0.01$) than hyporheic water and 1–2 pH units higher than groundwater pH during both seasons (Table 2). No significant differences were evident with depth or distance beneath the riffle.

Williams (1989) found little differences between surface and hyporheic specific conductance values for a hardwater Ontario stream. Station 2 surface water specific conductance averaged 197 μS/cm, while groundwater averaged 316 μS/cm (Table 2). Hyporheic specific conductance was higher than for surface water but did not significantly differ with depth into the bed; however, when values for all depths at each quadrat were composited, there was a significant decrease with distance beneath the riffle (Fig. 7; $p < 0.001$).

Total dissolved solids in hyporheic water were always higher ($p < 0.01$) than in surface water but not higher than in groundwater (Table 2). No consistent hyporheic patterns were apparent with either depth or distance beneath the riffle.

The hyporheic chemistry of the Maple River appeared to be quite similar to other North American systems. Compared with the limited number of studies, our results are in agreement for

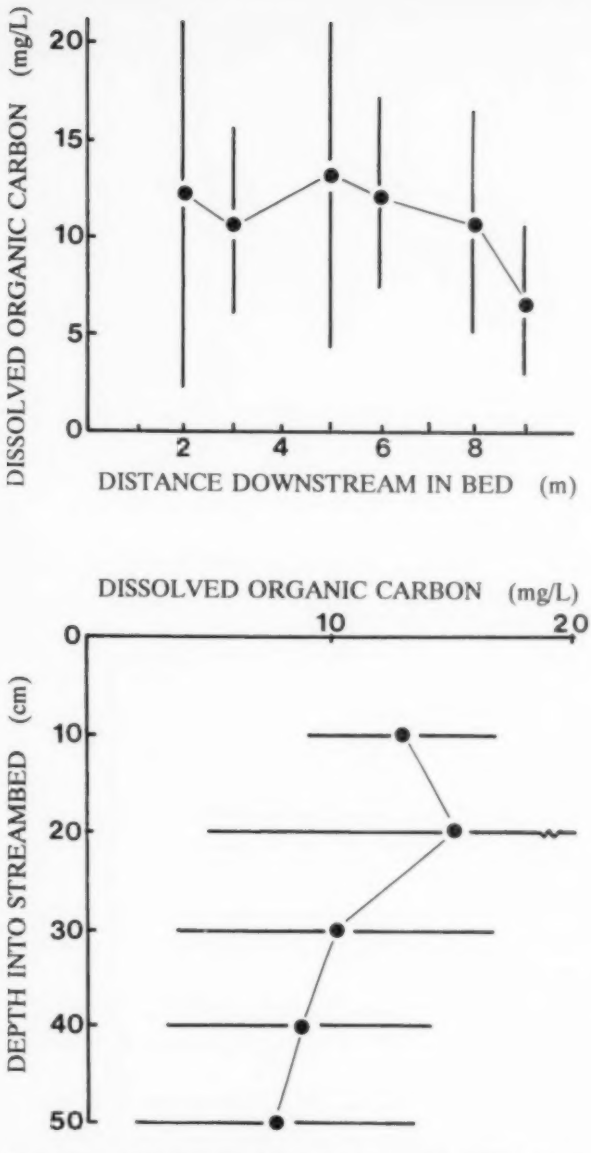

FIG. 6. Top; all-depth composite mean DOC concentrations (± 1 SD, n = 15) along the central longitudinal transect beneath the riffle at Station 2. Metre 1 is at the upstream end of the hyporheic zone and metre 9 is the downstream end. Bottom: all-distance composite mean DOC concentrations (± 1 SD, n = 21) with depth into the bed. All data from August 1987.

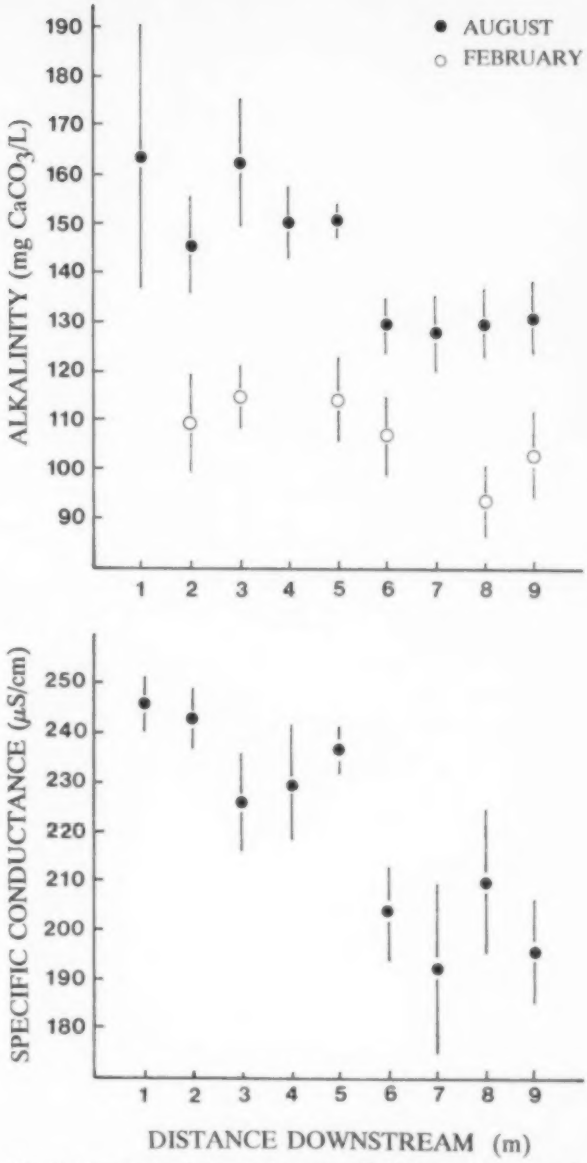

FIG. 7. Top: all-depth composite mean alkalinity (± 1 SD, n = 6) along the central longitudinal transect in the riffle at Station 2. Bottom: all-depth composite mean specific conductance (± 1 SD, n = 6) along the central longitudinal transect in the riffle at Station 2. Metre 1 is at the upstream end of the hyporheic zone and metre 9 is the downstream end. All data from August 1986.

virtually all parameters measured in vertical profiles from surface waters into deeper bed sediments. Differences between surface and groundwaters also appeared to be similar to those reported in other studies. Longitudinal profiles within hyporheic zones have not been reported previously but somewhat resembled lateral gradients beneath stream banks (Williams 1984, 1989). Based on vertical comparisons, we would expect analogous longitudinal gradients to occur in other systems, linked to geomorphologic and hydrologic variables.

Components less biologically active and with less affinity for sediments, such as temperature, chloride, and silica (Wetzel 1983), showed the strongest vertical and longitudinal gradients and came closest to approximating the Vaux (1962, 1968) and Thibodeaux and Boyle (1987) underflow models. The more biologically active constituents, such as nitrate, ammonium, SRP, and DO, followed expected longitudinal concentration shifts; however, vertical profiles did not because many of these, including DOC, were accumulated in the hyporheic zone, particularly in the upper 10–20 cm. Further, these constituents could be expected to exhibit a higher degree of patchiness in the hyporheic environment (Pringle et al. 1988), resulting from

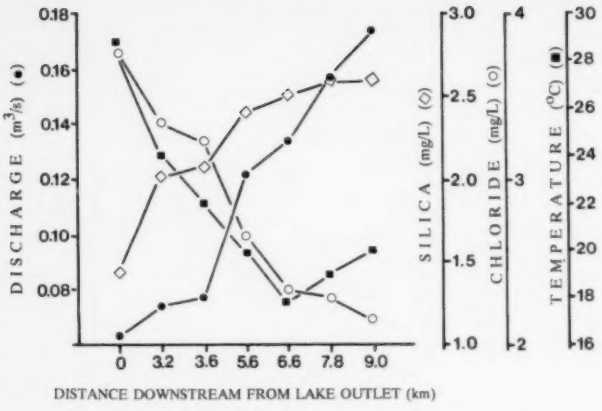

FIG. 8. Surface water discharge, temperature, silica, and chloride concentrations along a 10-km reach of the Maple River during base-flow conditions, August 1988. Error bars omitted for clarity. Silica, 1 SD $\leq \pm 0.01$; chloride, 1 SD $\leq \pm 0.02$; $n = 3$ for each river kilometre.

differential rates of uptake or production within the bed (Newbold et al. 1982; Elwood et al. 1983; Grimm and Fisher 1984).

In general, chemical patterns within the hyporheic zone beneath the riffle–pool segment appeared to be related to underflow patterns: the upstream end more resembling surface water and the downstream end more resembling both groundwater and interactions that had taken place within the bed. The coupling of advective and convective forces along the underflow paths may have encouraged surface water – groundwater mixing in the midsections and the possible return of mixed waters to the stream at the downstream end of riffles (see Bencala et al. 1984). Although yet to be tested, this would seem to have importance to solute transport, the distribution of hyporheic organisms, and sampling designs for hyporheic studies.

Whole-Reach Surface Water Patterns and Hyporheic Parallels

Summer base-flow surface water temperatures decreased downstream as discharge increased, e.g. from 29°C in August 1988 at the Douglas Lake outlet (Station 1) to 18°C at Station 5, with a slight increase to 20°C at Station 7 (Fig. 8). Decreases in temperature inferred groundwater inflow (discharge) over the whole reach. The temperature increases at Stations 6 and 7 in 1988 were suspected to be related to opening of the canopy, increased surface area, and increased retention time resulting from beaver impoundment (Naiman et al. 1986). Chloride concentrations decreased ($p < 0.01$) proportionately with increasing discharge downstream from 3.79 mg/L at Station 1 to 2.18 mg/L at Station 7 (Fig. 8). Conversely, silica (SiO_2) concentration increased ($p < 0.01$) with increasing discharge from approximately 1.45 mg/L at Station 1 to 2.60 mg/L at Station 7. The increase for silica, however, was not as linear as for chloride (Fig. 8), which may have reflected increased benthic production by diatoms, or an increased hyporheic zone depth which depressed groundwater transport of silica, again likely related to beaver impoundment (White 1990).

Discharge was not a good predictor in 1989 because of increased beaver activity between Stations 4 and 5 and Stations 6 and 7. Whole-reach nutrients were more related to river distance (kilometres) from Douglas Lake rather than to discharge at individual stations (Fig. 9). Using distance, a significant decrease ($p < 0.01$) occurred in whole-reach surface water SRP from 0.912 μg/L at Station 1 to below detection limits at and beyond Station 4. Maple River surface water SRP concentrations (1–6 μg/L) were comparable with eastern and boreal streams (Klotz 1985; Swank and Waide 1987; Ford and Naiman 1989; Stanford and Ward 1988). The largely aerobic substrata may act as a sink for phosphate, and regeneration back to surface water may not occur until reducing conditions prevail. SRP in some systems never reaches the substratum – surface water interface because of the high adsorption capacity of the substrata, particularly clays (Wetzel 1983). For example, first-order desert streams were not enriched by SRP from the hyporheic zone because of adsorption to the clay fraction of the substratum (D. L. Carr, University of New Mexico, Albuquerque, NM, pers. comm.). Maple River substrata contain little clay but instead are composed mostly of medium to fine sand, and some phosphate may be desorbed and exchanged to surface waters. Further, newly desorbed SRP may be used up immediately by periphyton or the microbial community at the sediment–water interface and consequently never reach the water column in detectable amounts.

A twofold increase occurred in nitrate concentration from 12.6 μg/L at Station 1 to 23.1 μg/L at Station 7 ($p < 0.01$) (Fig. 9). Surface water ammonium concentrations showed no relationship to distance from Douglas Lake or to discharge (Fig. 9). DOC decreased from 10.4 mg/L at Station 1 to 8.4 mg/L at Station 7 ($p < 0.01$) (Fig. 9). Differential uptake of surface water DOC (Lush and Hynes 1978b; Kaplan and Bott 1983; Mickelburgh et al. 1984) and groundwater DOC (Manny and Wetzel 1973) by the sediment microbial community may account, in part, for lack of parallels between hyporheic and whole-reach DOC patterns (Fig. 9). Chlorophyll *a* decreased from an average of 4.542 μg/L at Stations 1–3 to 3.068 μg/L at Station 7 ($p < 0.01$) and did not parallel hyporheic patterns (Fig. 9).

Temperature and chemical concentrations within the hyporheic zone showed many parallels with whole-reach surface water patterns. In general, surface water became less chemically similar to Douglas Lake water and more similar to hyporheic water/groundwater over the length of the reach. Longitudinal hyporheic and surface water patterns were quite similar for the more conservative properties such as temperature, chloride, and silica. More biologically active SRP, nitrate, and DOC also showed downstream surface water patterns which could be inferred from hyporheic patterns and groundwater concentrations. The disruptions in patterns at greater distances downstream from Station 2 probably were related to alterations in stream morphology and hydrology, i.e. beaver activity, and to biologically mediated processes within the stream or sediments.

Ford and Naiman (1989) were unable to correlate surface water DOC, nitrogen, phosphorus, or silica levels with potential groundwater input from seeps and concluded that inbed processes must have accounted for the differences. Their seeps were located some distance from the stream. Very little groundwater input appeared to come from springs or seeps in the Maple River, as we found little direct evidence of either. The more indirect route of entry through the hyporheic zone coupled with the initial differences between groundwater and Douglas Lake water allowed us to observe parallels between hyporheic and surface water patterns that otherwise might have been obscured.

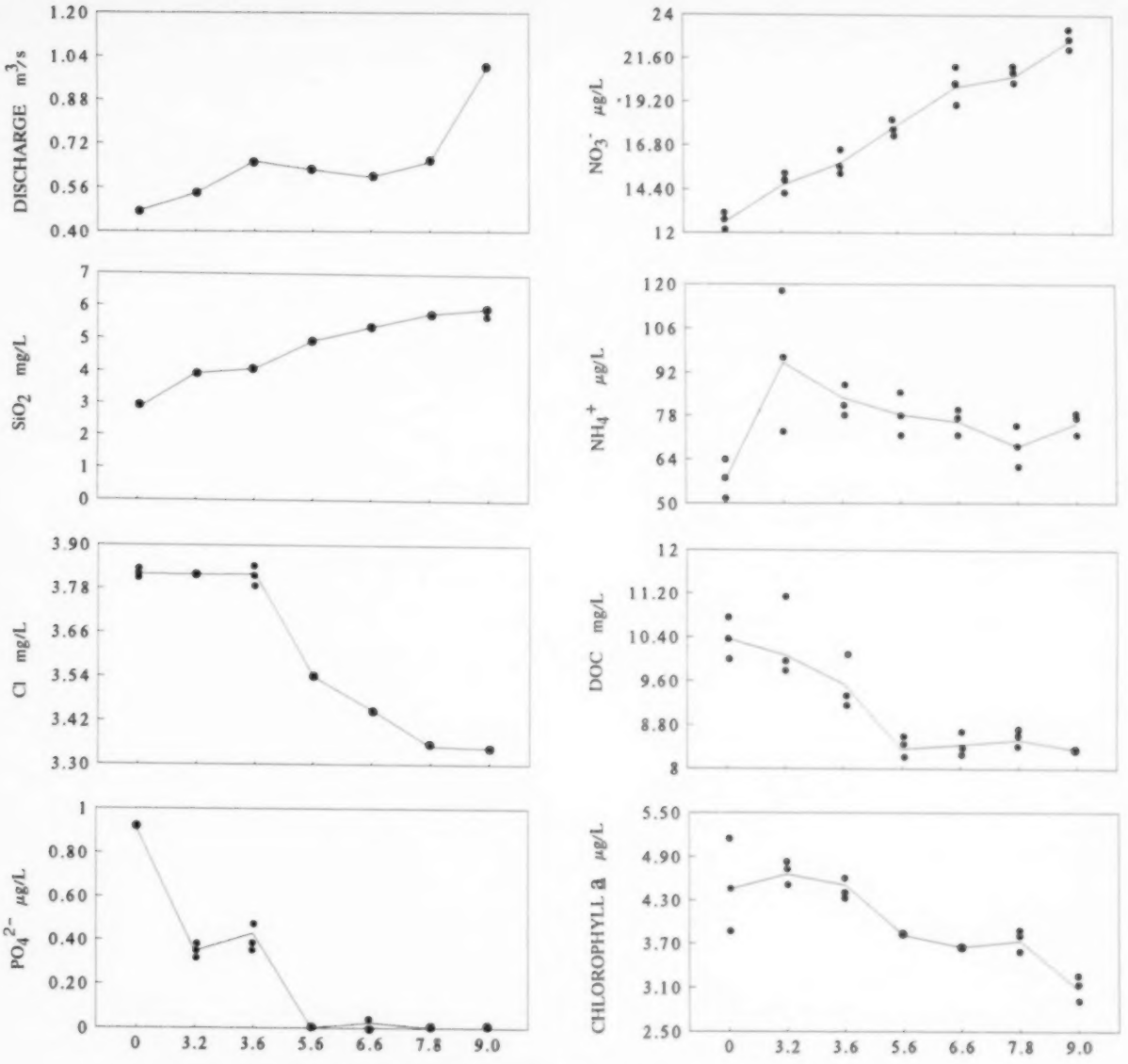

FIG. 9. Surface water discharge, silica, chloride, phosphate, nitrate, ammonium, DOC, and chlorophyll *a* along a 10-km reach of the Maple River during base-flow conditions, August 1989. All data points are plotted ($n = 3$).

Regardless of the groundwater entry route or even if groundwater is a contributing factor to surface flow, the hyporheic zone has been shown to be highly dynamic, acting as a sink and as a potential source and site of chemical processing. Patterns within a hyporheic zone appear to be predictable based on hydrologic principles, at least for the Maple River, and should not be overlooked in solute transport studies (Stream Solute Workshop 1990) or the overall nutrient budgets of stream ecosystems (Grimm and Fisher 1984; Ford and Naiman 1989).

Acknowledgements

We wish to acknowledge the University of Michigan Biological Station, particularly R. Glover and J. Houseal, for assistance with nutrient and DOC analyses, M. McGovern, C. Elzinga, L. Barnese, and J. Heinen for field assistance, and D. Deutschman for statistical assistance. D. Conley and D. Francis assisted with nutrient analysis at the Ann Arbor campus. K. Johnston, Hancock Biological Station, assisted with additional nutrient and DOC analyses. The University of Michigan Biological Station and Rackham School of Graduate Studies provided several grants to S.P.H. during the course of this study.

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A Polymorphism in the Ribosomal RNA Genes Distinguishes Atlantic Salmon (*Salmo salar*) from North America and Europe

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Cutler, M. G., S. E. Bartlett, S. E. Hartley, and W. S. Davidson. 1991. A polymorphism in the ribosomal RNA genes distinguishes Atlantic salmon (*Salmo salar*) from North America and Europe. Can. J. Fish. Aquat. Sci. 48: 1655–1661.

There is good evidence to suggest that Atlantic salmon (*Salmo salar*) from North America and Europe form distinct breeding populations. However, it has been very difficult to discover a genetic marker that allows the continent of origin of an individual salmon to be determined unambiguously. Repetitive elements in the nuclear genome have provided population-specific markers in several species. Therefore, we examined the ribosomal RNA gene (rDNA) complex in Atlantic salmon by Southern blot analysis using a cloned repeating unit of the *Drosophila melanogaster* rDNA as a probe. A restriction fragment length polymorphism that could distinguish between salmon from Newfoundland and Scotland was detected using a combination of the restriction enzymes *Sac*I and *Xba*I. The Newfoundland salmon had additional fragments of 3.6 kilobase pairs (kbp) and 2.6 kbp. Of 27 salmon from 10 populations in Europe, none possessed the 3.6- or the 2.6-kbp fragment whereas all 33 North American Atlantic salmon (from six populations) had the 2.6-kbp band and the majority showed the 3.6-kbp fragment. This preliminary survey strongly suggests that this genetic marker can be used to identify the continent of origin of individual Atlantic salmon.

Nous avons de bonnes raisons de croire que les populations de saumon de l'Atlantique (*Salmo salar*) de l'Amérique du Nord sont distinctes de celles de l'Europe et se reproduisent séparément. Il s'est toutefois révélé très difficile de trouver un marqueur génétique qui permette de déterminer de façon certaine de quel continent provient un saumon donné. Or, chez plusieurs espèces, des éléments répétitifs du génome nucléique ont servi de marqueurs de population. Nous avons donc étudié le complexe génique d'ARN ribosomique (ADNr) du saumon de l'Atlantique par transfert de Southern en utilisant comme sonde une unité répétitive clonée de l'ADNr de *Drosophila melanogaster*. Nous avons décelé trouvé un polymorphisme de longueur de fragment de restriction qui permet de faire la distinction entre le saumon de Terre-Neuve et celui de l'Écosse au moyen d'un mélange d'enzymes de restriction *Sac*I et *Xba*I. Le saumon de Terre-Neuve possède des fragments additionnels de 3,6 et 2,6 kilopaires de bases (kpb). Dans un ensemble de 27 saumons provenant de 10 populations d'Europe, aucun ne possédait le fragment de 3,6 ou 2,6 kbp, tandis que dans l'ensemble de 33 saumons provenant de six populations d'Amérique du Nord, tous avaient la bande de 2,6 kpb et la majorité, le fragment de 3,6 kpb. Les résultats de cette étude préliminaire indiquent que ce marqueur génétique peut servir à identifier le continent d'origine d'un saumon de l'Atlantique.

Received March 26, 1990
Accepted March 25, 1991
(JA522)

Reçu le 26 mars 1990
Accepté le 25 mars 1991

A comparison of the frequencies of protein electrophoretic variants in many Atlantic salmon (*Salmo salar*) populations has revealed that North American and European populations form two distinct breeding units (Ståhl 1987). However, it has been very difficult to use this procedure to tell the continent of origin of a salmon caught at sea. This is partly due to the small number of polymorphic loci available and to a lack of fixation of specific alleles in the different breeding units (see Davidson et al. (1989) for a review of genetic variation in Atlantic salmon). Only the plasma protein transferrin appears to have alleles that are associated with salmon from North America or Europe (Payne et al. 1971). Although the most common allele (T_f1) is found in all populations that have been examined, transferrin allele frequencies have been used to estimate the relative amount of North American and European salmon caught in the West Greenland commercial fishery (Payne 1980).

Atlantic salmon populations have also been screened for mitochondrial DNA restriction enzyme site polymorphisms (Birt et al. 1986; Palva et al. 1989; Gyllensten and Wilson 1987; Bermingham et al. 1988; Hovey et al. 1989; Birt et al. 1991). Bermingham et al. (1988) identified three restriction enzymes that gave different digestion patterns with mitochon-

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TABLE 1. Population analysis of the *Sac*I + *Xba*I ribosomal DNA polymorphism in Atlantic salmon.

Population	Salmon	Fragment length (kbp) 3.6[a]	2.6[a]
Bay D'Espoir,	A1	−	+
Hatchery R.,	A2	−	+
Newfoundland	A4	+	+
	A5	+	+
	A6	+	+
	A7	+	+
Conne R.,	B1	+	+
Newfoundland	B2	+	+
	B3	+	+
	B4	−	+
	B5	+	+
	B6	+	+
	B7	+	+
	B8	+	+
LaHave, R.,	C1	+	+
Nova Scotia	C2	−	+
	C3	+	+
	C4	−	+
	C5	+	+
	C6	+	+
	C7	−	+
	C9	+	+
Grey R.,	D1	+	+
Newfoundland	D2	−	+
	D3	+	+
	D5	−	+
Gambo Pond,	638	+	+
Newfoundland	666	+	+
(nonanadramous)	X13M	+	+
	X10B	+	+
Gambo Pond,	689	+	+
Newfoundland	910B1	+	+
(anadramous)	913	+	+
Lussa R.,	Lu1	−	−
Scotland	Lu2	−	−
Scourie Hatchery,	Sco1	−	−
Scotland	Sco2	−	−
	Sco3	−	−
	86/5	−	−
Uist,	U1	−	−
Scotland	U2	−	−
Marine Harvest,	MH1	−	−
Scotland	MH2	−	−
	MH/10	−	−
R. Dee, Scotland	Bell	−	−
R. Tummel, Scotland	Coll1	−	−
R. Carron, Scotland	Coll2	−	−
R. Earn, Scotland	Earn	−	−
R. Tweed, Scotland	TwSa1	−	−
Kame's Fish Farm,	K1	−	−
Scotland	K2	−	−
	K3	−	−
	K4	−	−
Almond R.,	Alm1	−	−
Scotland	Alm2	−	−
	A7	−	−
	A16	−	−
	B7	−	−
	B9	−	−
	08202	−	−

[a]Polymorphic bands as shown in Fig. 1. +, band present; −, band absent.

TABLE 2. Restriction fragments of Atlantic salmon DNA generated by various enzymes and observed after Southern blotting and probing with a *Drosophila melanogaster* rDNA repeat.

Restriction enzyme	Scottish Atlantic salmon fragment length(s) (kbp)	Newfoundland Atlantic salmon fragment length(s) (kbp)
*Bam*HI	2.8	2.8
*Bgl*II	5.2	5.2[b]
	2.9[a]	2.9[a,b]
*Eco*RI	7.6	7.6
	4.9	4.9
*Hind*III	10.6	10.6
	8.5	8.5
	2.0[a]	2.0[a]
*Kpn*I	8.7	8.7[b]
	2.7[a]	2.7[a,b]
*Pst*I	6.4	6.4[b]
	5.1	5.1[b]
*Pvu*II	6.7[a]	6.7[a]
*Sac*I	3.6	3.6
	2.3[a]	2.3[a,b]
	1.7[a]	1.7[a,b]
	1.1[a]	1.1[a,b]
*Xba*I	1.6[b]	1.6[b]

[a]Band was faint and difficult to observe on the autoradiograph.
[b]DNA appeared undigested when autoradiographed.

drial DNA from European or North American Atlantic salmon. The North American reference stocks that were used by these workers came from hatcheries and represented the southern range of the species. No samples from Newfoundland, Nova Scotia, or Quebec were examined. Recent studies (Birt 1990; Birt et al. 1991) have found that the European mitochondrial genotype described by Bermingham et al. (1988) is present in approximately 20% of Newfoundland salmon and it has been observed in salmon from Nova Scotia and Quebec (McVeigh et al. 1991).

A genetic marker from the nuclear genome would eliminate the concern associated with mitochondrial DNA markers. A second advantage of genomic DNA is that it is easily isolated from a blood sample and this avoids the necessity of killing a fish to sample it. Chromosomal studies by Phillips and Hartley (1988) have revealed that North American Atlantic salmon possess on average 54–56 chromosomes whereas 58 is the norm for European salmon. As this technique requires intact dividing cells, it is not a practical means for routine stock identification.

Repetitive elements in the nuclear genome offer the potential for population-specific genetic markers. The ribosomal RNA gene (rDNA) complex consists of multiple copies of a repeating unit that encodes the precursor of the 28 S, 18 S, and 5.8 S ribosomal RNAs. These gene products are highly conserved but the intergenic nontranscribed spacer region evolves rapidly in higher eukaryotes (Arnheim 1983). One form of evolution in rDNA results in changes in the spacer region which spread through an individual's genome and eventually members of an interbreeding population (Dover 1982). This creates intrapopulation homogeneity and interpopulation differences with respect to rDNA sequence and structure. Differences in the length of spacer both within individuals and between individuals have been reported for many species including humans (Krystal and Arnheim 1978) and salmonids (Popodi et al. 1985; Phillips et al. 1989). In addition, restriction fragment length polymorphisms in this region have been used to differentiate between populations of mice (Suzuki et al. 1986) and *Droso-*

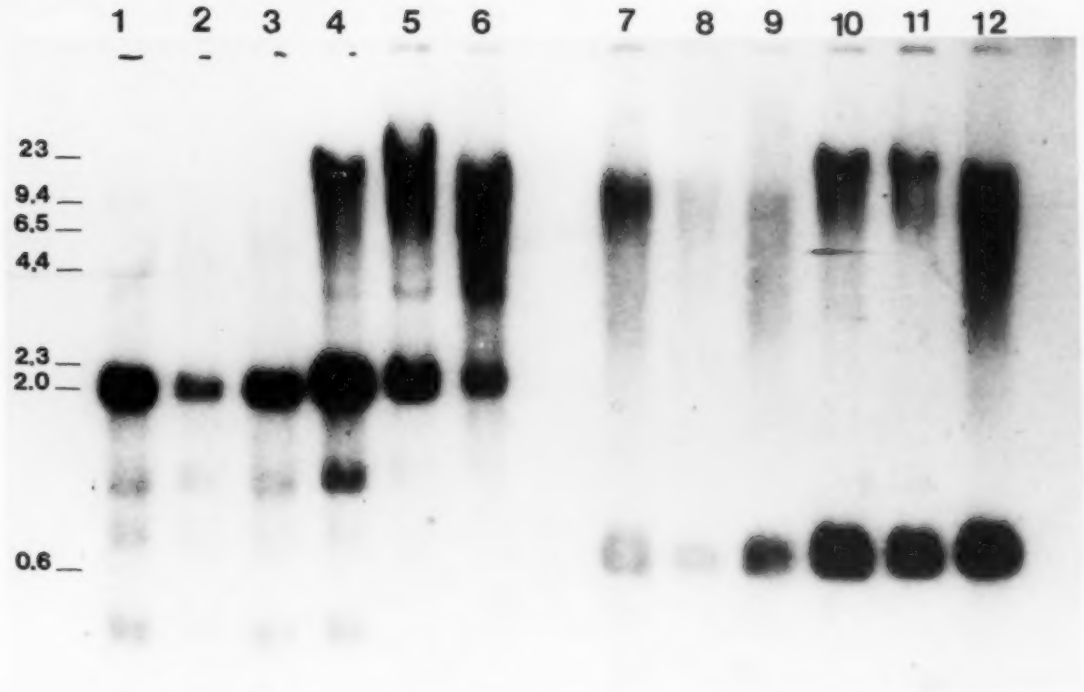

FIG. 1. Autoradiograph of Atlantic salmon genomic DNA digested with *Sac*I (lanes 1–6) or *Xba*I (lanes 7–12), Southern blotted, and probed with the *Drosophila melanogaster* rDNA clone. Scottish salmon: lanes 1 and 7 = A7, lanes 2 and 8 = B9, and lanes 3 and 10 = MH/10. Newfoundland salmon: lanes 4 and 10 = 689, lanes 5 and 11 = 913, and lanes 6 and 12 = 926B. (See Table 1 for the populations from which the salmon came.) Incomplete digestion of the Newfoundland salmon rDNA is seen with both enzymes whereas the rDNA of Scottish salmon is fully digested with *Sac*I.

phila (Williams et al. 1985). The purpose of this research was to examine the rDNA of Atlantic salmon from North America and Europe to determine if there were any restriction fragment length polymorphisms that could be used to tell these populations apart.

Materials and Methods

Salmon Populations

DNA was prepared from salmon from six North American and 10 European populations (Table 1). The North American populations consisted of three populations (Conne River, Grey River, and Hatchery Brook) from the south coast of Newfoundland, two sympatric populations (one anadromous, the other nonanadromous) from Gambo Pond, Newfoundland, and one population from LaHave River in Nova Scotia. The European salmon populations were from Lussa River, River Uist, River Dee, River Tummel, River Carron, River Earn, River Tweed, Almond River, Scourie Hatchery, Kane's Fish Farm, and Marine Harvest. All were Scottish in origin except for the Marine Harvest domesticated salmon that were derived from a Norwegian stock.

Restriction Enzymes

Enzymes were purchased from Pharmacia Ltd., Dorval, Quebec, and used according to the manufacturer's instructions. The restriction enzymes used in this study were *Bam*HI, *Bgl*II, *Eco*RI, *Hind*III, *Kpn*I, *Mlu*I, *Pst*I, *Pvu*II, *Sac*I, and *Xba*I.

rDNA Probe

The hybrid plasmid, pDM238, was a generous gift of J. Hancock, Genetics Department, Cambridge University, Cambridge, U.K. This plasmid contains the rDNA repeat of *Drosophila melanogaster* cloned into the *Eco*RI site of the cloning vector pBR322. The rDNA insert was isolated as an 11.7 kilobase pair (kbp) fragment by preparative gel electrophoresis using "Geneclean" (Bio/Can Scientific, Mississauga, Ontario). Digestion of the *Eco*RI insert with *Mlu*I produced two fragments that could be used to identify the 18 S and 28 S regions of the rDNA complex.

Salmon DNA

Salmon DNA was prepared from whole blood or liver by a procedure involving proteinase K digestion in the presence of SDS, a phenol–chloroform extraction, treatment of the crude nucleic acid with heat-treated ribonuclease, and finally ethanol precipitation. The DNA was dissolved in 10 mM Tris/HCl, 1 mM EDTA, pH 8.0, and stored at 4°C.

Southern Blotting

Salmon DNA (2 μg) was digested overnight with the appropriate restriction enzymes and subjected to electrophoresis in

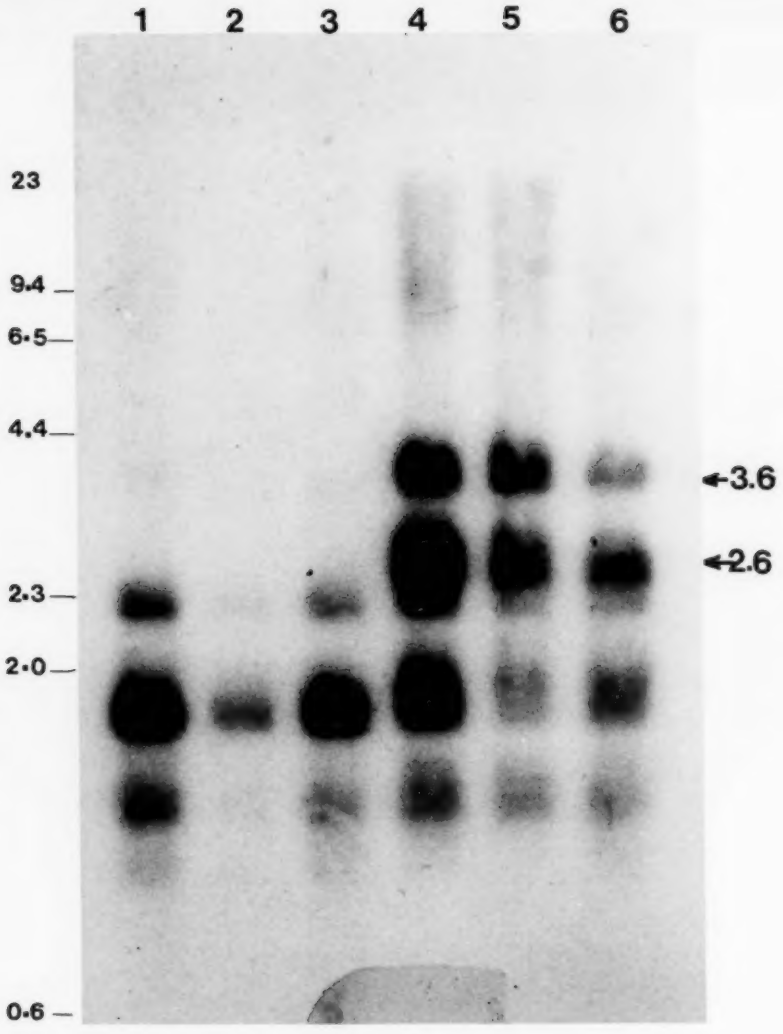

FIG. 2. Autoradiograph of Atlantic salmon genomic DNA showing the restriction fragment polymorphism patterns produced when the DNA is digested with *Sac*I and *Xba*I, Southern blotted, and probed as in Fig. 1. The additional fragments of size 3.6 and 2.6 kbp are seen in the Newfoundland samples (lanes 4–6) and are indicated by the arrows. The Scottish samples are in lanes 1–3. The numbers referring to the particular samples are the same as in Fig. 1.

0.8% agarose gels (14 cm long) in a Tris/borate/EDTA buffer at 3V/cm for 3 h. The DNA was visualized by staining with ethidium bromide and a photograph of the gel was taken. λ DNA cut with *Hind*III served as size standards. The DNA was denatured in situ and transfered to Hybond N nylon membranes (Maniatis et al. 1982).

Hybridization Conditions

The nylon membranes were prehybridized with 10 mL of a solution consisting of 6 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M trisodium citrate), 50% formamide, 5 × Denhardt's solution (1 × Denhardt's solution contains 0.02% bovine serum albumin, 0.02% ficoll, and 0.02% polyvinylpyrrolidone), 50 mM phosphate buffer, pH 6.5, and 0.5% SDS for 3 h at 37°C. The probe was labelled with [α^{32}P]dCTP to a specific activity of approximately 10^8 dpm/μg by random priming using a kit from Bethesda Research Lab., Burlington, Ontario. Each membrane was hybridized with approximately 2×10^6 dpm of probe in 5 mL of the same solution that was used in the prehybridization step. Hybridization continued for 12–16 h at 37°C. After hybridization, the membranes were washed three times at room temperature in 250 mL of 2 × SSC, 0.1% SDS for 15 min each, followed by a single wash in 250mL of 2 × SSC, 0.1% SDS at 55°C, and three washes in 1 × SSC, 0.1% SDS for 20 min at 55°C. The membranes were air dried and exposed to X-ray film with an intensifying screen at −70°C for 2–3 d prior to developing the film.

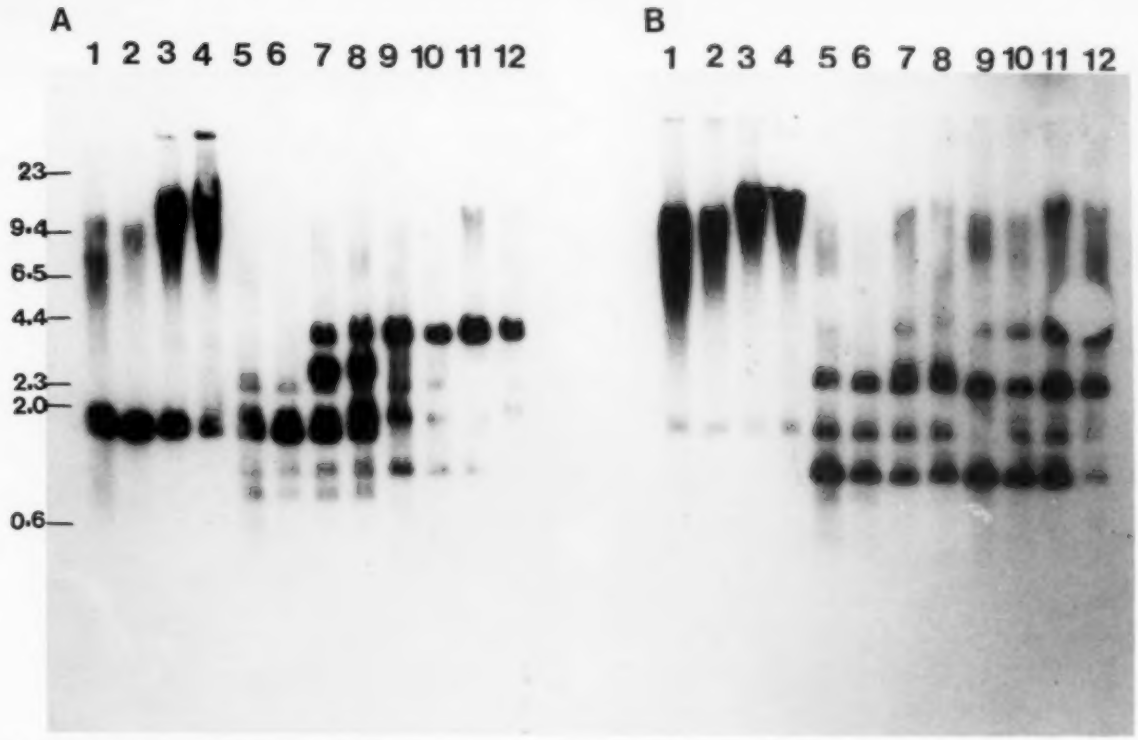

FIG. 3. A pair of Scottish salmon genomic DNAs (lanes 1 and 2, 5 and 6, and 9 and 10) and a pair of Newfoundland salmon genomic DNAs (lanes 3 and 4, 7 and 8, and 11 and 12) were digested with *Xba*I (lanes 1–4), *Sac*I plus *Xba*I (lanes 5–8), and *Sac*I (lanes 9–12). Southern blot analysis with an 18 S specific probe (A, 1–12) or a 28 S specific probe (B, 1–12) revealed that the restriction fragment length polymorphism is in the 18 S region of the salmon rDNA. The faint bands in the autoradiographs are a result of cross-contamination of the probes.

Results

Single Restriction Enzyme Digests

Phillips et al. (1989) have constructed a restriction map of the Atlantic salmon rDNA repeat and identified variable sites for *Eco*RI, *Bam*HI, and *Pst*I in the nontranscribed spacer region. These sites were found to vary between and within individuals. Six Atlantic salmon genomic DNAs, prepared from three Scottish salmon and three Newfoundland salmon, were digested with a panel of restriction enzymes and subjected to Southern blot analysis with the cloned *Drosophilia* rDNA repeat as a probe. The fragment sizes produced by each of the restriction enzymes are given in Table 2. No restriction fragment length polymorphism was evident among any of the salmon. However, the Newfoundland salmon DNAs appeared to be digested less than the Scottish salmon DNAs with *Bgl*II, *Kpn*I, *Pst*I, *Sac*I, and *Xba*I. It was unlikely that this was due to the presence of an inhibitor or because of incomplete digestion. Rather, it suggested that some of the sites for these enzymes were lacking in a subset of the rDNA in the Newfoundland salmon. Another observation that supported this notion was the difference in the relative intensities of some of the bands on the autoradiographs.

Identification of a Genetic Marker

Genomic DNA containing poorly digested rDNA was present, especially in the Newfoundland salmon samples (Fig. 1). Similarly, the faint high molecular weight bands present in the Newfoundland samples digested with *Sac*I suggested that there are variable sites for this enzyme in Newfoundland salmon but not in Scottish salmon. The *Xba*I analysis gave incomplete digestion in both groups, suggesting that there may be variable sites. Double digestion of the six salmon DNAs with *Sac*I and *Xba*I and subsequent probing with the pDM238 insert revealed a restriction fragment length polymorphism between these groups of salmon. Newfoundland salmon have two additional fragments (3.6 and 2.6 kbp) compared with the Scottish samples (Fig. 2). In addition, there was little undigested DNA observable in either group after the double digest.

Regional Localisation of the Variable Sites within the rDNA

To determine the approximate locations of the variable sites within the rDNA complex, two Scottish and two Newfoundland salmon DNAs were digested with *Sac*I, *Xba*I, and *Sac*I and *Xba*I together and Southern blot anlaysis was carried out using the 18 S region or the 28 S region of the cloned *Drosophila* rDNA as probes. The polymorphic sites lie in the 18 S region of the salmon rDNA complex (Fig. 3). However, for practical purposes it was more convenient to use the entire cloned fragment when surveying populations.

Survey of Atlantic Salmon Populations

As the restriction fragment length polymorphism cosegregated with the continent of origin in the six salmon analyzed initially, a population survey was carried out to determine the

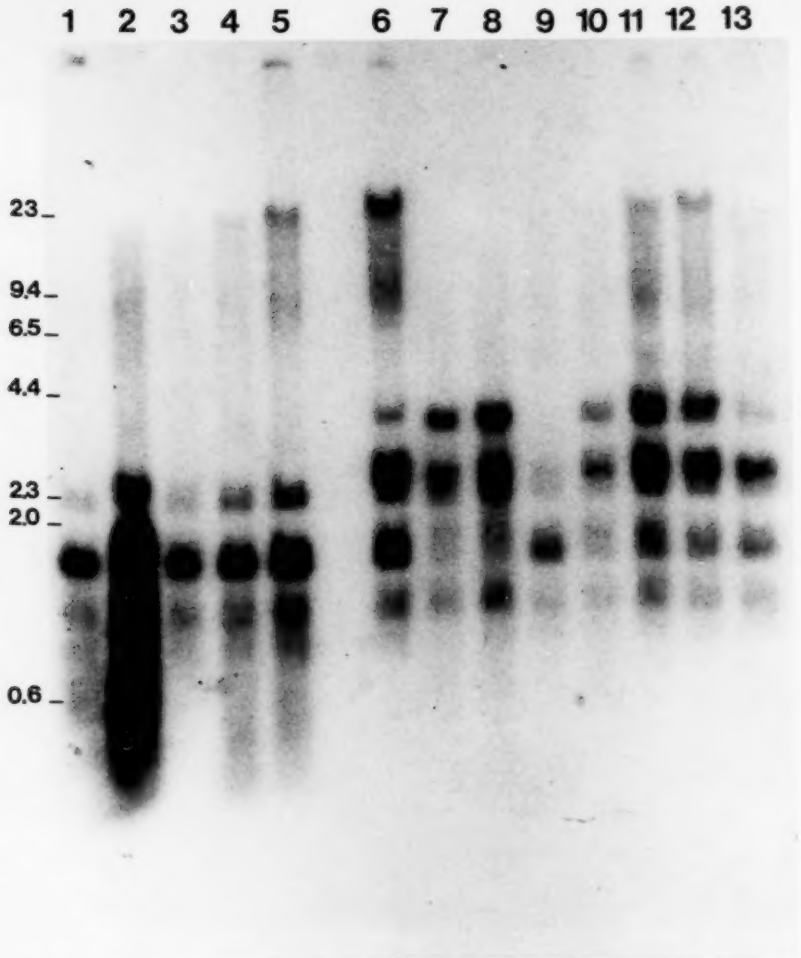

FIG. 4. Autoradiograph showing that the restriction fragment polymorphism patterns cosegregate with the continent of origin for several populations on both sides of the Atlantic. Only the North American fish display the 3.6- or 2.6-kbp bands. European salmon: lane 1, U1; lane 2, U2; lane 3, 86/5; lane, 4 MH/1; lane 5, MH/2. Lanes 6–13 are B1–B8, respectively, from Conne River, Newfoundland. The origin of the salmon is given in Table 1.

distribution of the genetic marker between the two continental groups of Atlantic salmon. A total of 33 genomic DNAs from six populations of North American Atlantic salmon and 27 genomic DNAs from 10 populations of European salmon populations were examined (Fig. 4). All of the North American salmon displayed at least one of the polymorphic bands (Table 1). The 2.6-kbp band was seen in every one of the North American Atlantic salmon whereas the 3.6-kbp fragment was observed in 76% of these fish. The inability to detect the 3.6-kbp fragment in some of the North American samples may have been due to the partly degraded nature of some of these DNAs. Neither the 3.6- nor the 2.6-kbp fragment was observed in any of the European samples. These results strongly suggest that this rDNA genetic marker can be used to determine the continent of origin of an individual Atlantic salmon.

Discussion

The restriction map of the Atlantic salmon rDNA repeat proposed by Phillips et al. (1989) does not appear to correspond to the results that were obtained in this study. This discrepancy is most likely due to a difference in the hybridization probes employed. Phillips et al. (1989) used a mouse 28 S rDNA and a hamster 18 S rDNA to define these regions of the map whereas the *Drosophila* probe used in this study encompasses both the 18 S and the 28 S genes. As only the coding regions of the rDNA repeat are highly conserved through evolution, these probes probably do not cross-hybridize with the most variable spacer regions. These sequences are only detected if they are associated with the conserved 18 S or 28 S coding sequences. Single restriction enzymes failed to detect any useful genetic marker but suggested that variable restriction sites exist. A combination of *Sac*I and *Xba*I proved capable of revealing a difference in the rDNAs of Newfoundland and Scottish salmon. This preliminary survey was extended to include 33 North American and 27 European salmon and the cosegregation of the genetic marker and continent of origin held true.

Although there are many populations of salmon on both sides of the Atlantic that have not been examined yet, there is a strong

indication that this genetic marker will prove very useful for determining the continent of origin of individual salmon. It has been shown that Atlantic salmon from the southern range of North America have different mtDNA genotypes from European salmon (Bermingham et al. 1988). It appears that it is only some Atlantic salmon from the northern range of North America (in particular Newfoundland and Quebec) that contain the "European" mitochondrial genotype (McVeigh et al. 1991). As these populations have already been screened for the "continent-specific" rDNA polymorphism and there was no evidence for the "European" pattern, it is likely that the results from our preliminary survey will be representative of the extended survey that we are in the process of conducting.

Acknowledgements

We thank J. Hancock for pDM238, T. Birt, V. Pepper, J. Thorpe, and several others for providing salmon blood, liver, or DNA, and T. Birt, V. Friesen-Birt, H. McVeigh, and R. Phillips for valuable discussions. Funding for this research came from NSERC (strategic grant to W.D.S. and undergraduate summer scholarship to M.G.C.), DFO (subvention grant and contract to W.S.D.), and NERC (grant to S.E.H.).

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Scale Morphology as an Index of Developmental Stability and Stress Resistance of Tilapia (*Oreochromis niloticus*)

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Shackell, N. L., and R. W. Doyle. 1991. Scale morphology as an index of developmental stability and stress resistance of tilapia (*Oreochromis niloticus*). Can. J. Fish. Aquat. Sci. 48: 1662–1669.

Growth and the stabilities of growth and development were examined in inbred, outbred, and interstrain hybrid lines of tilapia (*Oreochromus niloticus*) under two diet regimes. Developmental stability was quantified as the inverse of the number of deformed scale circuli (calcified ridges). Growth stability was measured as the inverse of the individual variability of circulus spacing (circulus spacing is an indirect measure of growth rate). Each line was fed an alternating diet of low-protein rice bran and high-protein commercial pellet or a constant diet of commercial pellet. Diet regime had a significant effect on growth, developmental stability, and growth stability. Genetic composition of line had a significant effect only on developmental stability. Inbred lines had lower developmental stabilities than outbred lines. Hybrid progeny had higher developmental stability than the average of the parent strain values. Faster growing individuals had higher developmental and growth stabilities and lower growth stabilities (only within the commercial pellet regime). Composite indices of "merit" can be constructed by combining the variables, according to the correlations among variables and breeding objectives.

On a examiné la croissance et la stabilité de la croissance et du développement chez des lignées consanguines, croisées et hybrides intersouches de tilapia (*Oreochromus niloticus*) soumis à deux régimes alimentaires différents. On a quantifié la stabilité du développement comme l'inverse du nombre des stries d'accroissement déformées (crêtes calcifiées). La stabilité de la croissance a été calculée comme l'inverse de la variabilité individuelle de l'espacement des stries d'accroissement (cet espacement est une mesure indirecte du taux de croissance). On a administré à chacune des lignées soit une alternance de nourriture à base de son de riz à faible teneur en protéines et de boulettes commerciales à forte teneur en protéines, soit de façon constante des boulettes commerciales. Le régime alimentaire a eu un effet significatif sur la croissance, la stabilité de la croissance et la stabilité du développement. La composition génétique de la lignée a eu un effet significatif seulement sur la stabilité du développement. Les lignées consanguines présentaient des stabilités de croissance plus faibles que les lignées croisées. La progéniture hybride avait une stabilité de développement plus élevée que la moyenne des valeurs chez les souches parentes. Les individus à croissance plus rapide présentaient des stabilités de croissance et de développement plus élevées. Les familles à croissance plus rapide avaient des stabilités de développement plus élevées et des stabilités de croissance plus faibles (seulement avec le régime à base de boulettes commerciales). On peut construire des indices composés de "mérite" en combinant les variables selon les corrélations entre les variables et les objectifs d'élevage.

Received August 31, 1990
Accepted March 15, 1991
(JA710)

Reçu le 31 août 1990
Accepté le 15 mars 1991

Genetic improvement programs in aquaculture are not yet a research priority and have a short history relative to programs in agriculture and animal husbandry (Pullin and Capili 1988; Lannan et al. 1989). Increased growth rate is usually the primary objective during the initial stage of genetic improvement programs (Jinks and Pooni 1988). However, agricultural programs show that stress and disease resistance become equal in importance to growth as genetic gains are achieved (Plucknett and Smith 1986). In South East Asia, rice varieties selected for high yield succumb more readily than primitive varieties to diseases and pests. These varieties require a monoculture system with minimal environmental variation, which can only be achieved by expensive maintenance management involving large amounts of chemical fertilizers and pesticides (Plucknett and Smith 1986). Plant geneticists are now acknowledging the importance of stress resistance by selecting for growth stability across a range of environments (statistical methods in Eberhart and Russell 1966; Hanson 1970; Westcott 1986; Wolff and Van Delden 1987; Witcombe 1988; Ariyo 1990).

Aquaculture genetic improvement programs are at an earlier stage, and superior growth is almost always the principal objective. Using the hard-won experience of agriculturists, fish breeders should consider selecting fast-growing fish that are also stress resistant (Chevassus and Dorson 1990). The importance of foreseeing, and perhaps forestalling, future problems is especially great in developing countries, where fish farms tend to be variable and poorly controlled. The geneticist's objective may then be to develop fish that are capable of good, reliable growth in poorly understood, poorly controlled, and fluctuating ecosystems. The criterion of "merit" in the selection program might then incorporate both growth and the reliability of growth.

If merit can be operationally defined, it can be measured and used in a selection program. In this paper, we use scale morphology to characterize the contribution of growth, developmental stability, and growth stability to the overall merit of tilapia (*Oreochromus niloticus*). Developmental stability (ability to regulate development in order to produce an optimal body

TABLE 1. Mating design of Nifi and Israel strains used in the experiment. Inbreeding coefficient F is defined as the increment in inbreeding above the preexisting (unknown) level in the population (Falconer 1989).

	Inbred	Outbred	Hybrid
Nifi	4	4	
Israel	4	4	
Israel mothers × Nifi fathers			4
Nifi mothers × Israel fathers			4
Inbreeding coefficient (F)	0.25	0	0

structure) is quantified as the inverse of the number of deformed circuli (calcified ridges on the scale) per individual. Growth stability (ability to maintain constant growth rate during environmental fluctuations) is measured as the inverse of individual variability of spacing between circuli. For the purposes of this paper, we equate insensitivity to fluctuations in dietary quality with "stress resistance" or "performance stability" as these concepts are used in terrestrial agriculture. We examined growth, developmental stability, and growth stability of inbred, outbred, and interstrain hybrid tilapia reared under two different diet regimes. We also examined the relationships among growth and developmental and growth stabilities at the individual and family levels, in the context of their implications for aquaculture bioeconomics and the evolutionary genetics of tilapia. Both economic and evolutionary strategies of genetic improvement are combinations of fast or slow growth and high or low developmental and growth stabilities. The preferred bioeconomic strategy is dependent on the underlying genetic correlations and on breeding objectives. The provisional bioeconomic assumption of this paper is that fast growth and high developmental and growth stabilities are desirable traits in aquaculture systems in developing countries. We do not propose this as a universal strategy but use it to demonstrate how the three criteria can be combined.

Material and Methods

Genetic Composition of Lines

The tilapia used in this study were derived from two strains, "Nifi" and "Israel" (Z. Basiao, SEAFDEC, Rizal, Philippines, pers. comm.), maintained at Binangonan Freshwater station in the Philippines. Breeding sets of one male and three females were placed in circular fibreglass tanks and fed ad libitum. When one female had produced a brood, the male and females were replaced into the base population. Only one fullsib family was used from each set of male/female matings. Inbred families were produced by one generation of brother/sister matings. Outbred and hybrid families were produced by mating unrelated parents. The full mating design involved offspring from 24 fullsib families (Table 1).

Experimental Methodology

One hundred fry were randomly selected from each family when approximately 4 wk old. Fifty fish were allotted to fluctuating environment (FE) groups, and 50 fish were allotted to constant environment (CE) groups. The FE groups were fed rice bran from week 0 to week 2, commercial food pellet from week 2 to week 4, and rice bran from week 4 to week 6. The

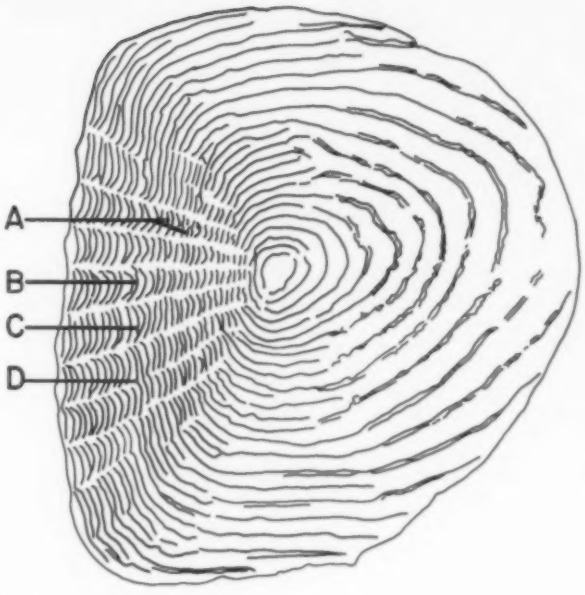

FIG. 1. Tilapia scale showing deformed circuli. Anterior margin (left-hand side) is the growing edge. Types of deformation: A = deposited incompletely; B = overextended into the intersector space; C = fused with adjacent circuli; D = extended into the next sector to form a band across the anterior portion of the scale. Circuli may also be irregularly deposited, resulting in a partially inverted circulus similar to type A.

CE groups were fed commercial food pellet throughout the experiment. Each group (48 groups of 50 fish each) was placed in a 250-L aerated polyethylene tank. Feces and debris were removed and one third of the water was changed daily in the tanks. Rice bran has a lower protein content (6.61%) than commercial food pellet (26%) and is known to suppress growth in milkfish (*Chanos chanos*), relative to other artificial or natural diets (Santiago et al. 1989). At the end of the experiment, 40 fish were sampled from each regime/line/family combination (four families in six lines at two regime levels). Standard length and weight were measured. Scales were sampled one row above the upper lateral line and three rows back from the head and preserved in 10% buffered formalin.

Growth Measure

Standard length, measured at 0 and 6 wk, was used to estimate growth rate directly. Percent specific growth rate per day was estimated as

((Individual log standard length (mm) at week 6 − family mean log standard length (mm) at week 0)/number of days) × 100).

Note that individual starting lengths were not obtained so the growth estimates of individuals were subject to some error.

Scale Measurements

The structure of the anterior, or growing, edge of the scale does not change after it has formed, so the scale serves as a record of past development and growth (van Oosten 1957; Sire 1986). Scale growth has often been used as an estimate of change in fish length (Doyle et al. 1987; Weatherley and Gill

1987; Barber and Walker 1988; Galloway and Kilambi 1988), but additional scale characteristics such as mode of formation and microstructure have not been as thoroughly researched. As early as 1931, Gray and Setna recorded that circuli (the bony concentric ridges on fish scales) were deformed in semistarved fish as compared with fish fed to satiation. Reproduction and cold temperatures also cause the formation of irregular circuli and are often referred to as "checks."

The anterior portion of the tilapia scale (Fig. 1) is the growing edge which is divided into 2–10 individual sectors. Circuli, ossified ridges, are deposited as the scale grows. The usual formation of circuli appear as crescent-shaped ridges, evenly spaced along a sector. When a fish is stressed, the uniformity of circulus formation is disrupted and/or the even spacing between circuli becomes irregular. These two phenomena were used to quantify abnormal development of the scale.

Developmental Stability: Circulus Deformation

We refer to the scale morphology measure of developmental stability as "deformation score." The similarity among repeated developmental events is a measure of developmental stability as the same genes express themselves again and again. It is assumed that repeated structures are formed through the activity of the same genes and that an abnormal structure results because the expression of genes was affected by internal and/or external environments (Falconer 1989). Falconer (1989) referred to a decrease in repeatability of developmental events as a measure of developmental noise. For example, Reeves (cf. Falconer 1989) studied the repeatability of abdominal bristles on *Drosophila melanogaster*. Reeves used the amount of variation between repeated events of bristle formation as a measure of developmental variation arising from the environment. In our case, circulus deposition on scales is a repeated developmental event; deformations are an indication of developmental noise. The underlying concepts of repeatability are similar to those underlying the concept of fluctuating asymmetry. Fluctuating asymmetry is a measure of the magnitude of asymmetry of bilateral organisms and is an established procedure used to detect epigenetic stress (Palmer and Strobeck 1986; Leary and Allendorf 1989; Parsons 1990). Essentially, fluctuating asymmetry is the repeatability of development between two sides of the body.

When tilapia undergo an environmental change, for example transfer from hatchery to fish pond, or from fresh to saline water, scale circuli are often laid down abnormally, e.g. circuli are broken, fused, or incomplete. We take this to be an indication of stress resulting from the change, or subsequent to it. The type of stress is not recorded on the scale, but the severity of stress can be measured by the amount of circulus deformation. In this study, the abnormality of circulus formation was quantified and used as an inverse index of developmental stability across environments.

The various types of deformation were categorized and are labelled in Fig. 1. Deformations were scored on the innermost five sectors: the central sector and the two sectors on each side of the central sector. The magnitude of deformation was measured by scoring the total number of deformed circuli on each scale. Data were log-transformed prior to analysis to minimize the effect of scale size and to equalize variances between regimes.

Growth Stability: Individual Variability of Growth

The measure of growth stability we refer to as "individual variability of growth." Fish with relatively wider spacing between circuli have higher growth rates (Doyle et al. 1987). When the rate of growth is decreased, the spacing between circuli is either reduced or circuli are not completely laid down (Bilton and Robins 1971a, 1971b). Similarly, when conditions allow for an increase in growth, circulus spacing becomes wider. Individual variability of growth was measured as follows. Circulus spacing was measured at seven radial distances along the central sector of the scale. Individual variability of growth was estimated simply by calculating the variance of seven circulus spacings on each individual. Data were log-transformed prior to analysis due to underlying chi-squared distribution of variances and to equalize variances between regimes. Scales were wet-mounted on glass slides and measured on a Leitz Projection microscope at 100×. Two scales per fish, 15 fish per each regime/line/family combination were measured. The average of the two measurements per fish was used in the subsequent analyses.

Composite Index of Overall Merit

A composite index of individual merit was constructed based on our operational definition of merit. Deformation score is entered inversely in the index, as it is a measure of the loss of integrity of development and a decrease in developmental stability. Individual variability of growth is entered inversely, as it means that the growth of a genotype is relatively sensitive to environmental fluctuations. Accordingly, a high merit value represents a relatively fast growing fish, with high developmental stability (low deformation score) and a stable pattern of growth (low individual variability of growth). Since there is as yet no empirical information on the relative economic value of the component variables, the component variables were standardized so they contributed equally to the variance of the index.

TABLE 2. ANOVAs of four variables (growth, deformation score, individual variability of growth, and composite merit) with two factors, regime (two) and line (six), with families (four) nested within line (FwL). $N = 760$; df = degrees of freedom, MS = mean square, and P = probability.

		Growth ($r^2 = 0.580$)		Deformation score ($r^2 = 0.428$)		Variability of growth ($r^2 = 0.176$)		Composite merit ($r^2 = 0.501$)	
Source	df	MS	P	MS	P	MS	P	MS	P
Regime	1	5.761	<0.001	24.380	<0.001	7.118	<0.001	1210.452	<0.001
Line	5	0.743	n.s.	1.401	<0.01	0.847	n.s.	49.538	<0.01
FwL	18	0.322	<0.001	0.260	<0.001	0.453	<0.001	10.438	<0.001
Error	735	0.015		0.066		0.128		2.195	

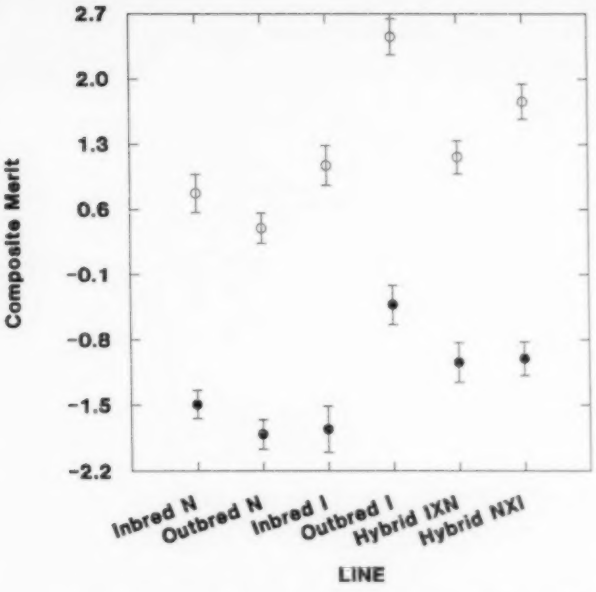

FIG. 2. Composite merit (sum of growth, inverse number of deformed circuli, and inverse variability of growth) plotted against line. Each point is mean score of four families per line, within regime (solid symbols = variable and less nutritious regime FE; open symbols = constant and more nutritious regime CE). Error bars are standard error of the mean. I = Israel strains; N = Nifi strain.

Ideally, the components of merit indices used in selection programs should be weighted by heritabilities and relative economic values (Falconer 1989, p. 326). Our index is merely designed to demonstrate how three important criteria can be combined to assess overall merit. The index was calculated using the following equation.

Merit = standardized growth
− standardized deformation score
− standardized individual variability of growth.

In particular, we were curious as to whether growth was positively or negatively correlated with the other two index components. Positive genetic correlations signify potential trouble for stock improvement programs. In fact, growth instability was inadvertently selected during the development of high-yielding rice crops (Plucknett and Smith 1986). It should be noted that in this study it was clear that individual variability of growth would be higher in regime FE because the regime was changed from rice bran to commercial food pellet for an intermediate period of 2 wk and then shifted back to rice bran. The CE group was fed a constant diet of commercial food pellet. The mean variability of growth was naturally higher in regime FE because individuals had to adjust to the change in type of diet.

Statistical Analyses

A factorial analysis of variance (ANOVA) with sampling was used for the main analyses of four variables: growth, deformation score, individual variability of growth, and standardized composite merit. The factors are regime and line, and families are nested within line:

TABLE 3. Line means of pooled regimes for four variables (n = 8 families; (± SD) in parentheses).

	Growth (mm)	Deformation score	Variability of growth	Composite merit
Inbred Nifi	0.727	1.205	0.181	−0.324
	(0.213)	(0.280)	(0.360)	(1.845)
Outbred Nifi	0.650	1.134	0.252	−0.707
	(0.156)	(0.266)	(0.263)	(1.671)
Inbred Israel	0.727	1.106	0.299	−0.331
	(0.170)	(0.361)	(0.428)	(2.317)
Outbred Israel	0.857	0.958	0.259	0.926
	(0.166)	(0.342)	(0.453)	(2.174)
Hybrid I × N	0.789	0.984	0.433	0.033
	(0.150)	(0.299)	(0.360)	(1.896)
Hybrid N × I	0.820	1.000	0.346	0.377
	(0.168)	(0.355)	(0.392)	(2.013)

$$Y_{ijkl} = \mu + R_i + L_j + F_{k(j)} + E_{ijkl}$$

where Y is individual observation of one of the four variables (growth, deformation score, individual variability of growth, merit), μ is the population mean, R is the effect of ith regime (two levels), L is the effect of the jth line (six levels), F is the effect of the kth family (four families) nested within the jth line, and E is the random error term of individual fish (average 16/cell). A line × regime interaction term was included in the above model to check for dissimilar response of lines to level of regime.

Results

Effect of Regime and Line

The effects of regime and family nested within line were significant in all four analyses. The effect of line was significant in the analyses of deformation score and composite merit, but nonsignificant in the analysis of growth and individual variability of growth (Table 2; Fig. 2). The effect of line in the analysis of deformation score was significant, although small relative to the effect of regime. The significant effects of regime and line were further analysed with an interaction term in the ANOVA to see if the response of lines to level of regime was similar. The interaction term was nonsignificant, indicating that lines responded similarily to the dietary regime.

Overall, the mean deformation scores of the inbred Nifi and Israel lines were 6.26 and 15.45% higher than the corresponding outbred lines. The means of the hybrid Israel × Nifi and Nifi × Israel were 5.93 and 4.40% less than the average of outbred Nifi and Israel. The line mean score of outbred Nifi was 18.37% higher than outbred Israel (Table 3).

Effect of Strain and Cross

To determine the effect of inbreeding, hybrids were dropped from the analysis and lines were recategorized according to level of inbreeding (cross) and their strain of origin (strain). Data were analysed using ANOVA with three factors: regime, strain, and cross. The effect of regime was significant in analyses of all four variables. The effects of cross and strain were significant in the analysis of all variables except individual variability of growth (Table 4). The interaction terms indicated that inbred groups had a similar response to level of dietary regime, that strains responded dissimilarly with respect to deformation scores to level of dietary regime, and that the growth response

TABLE 4. ANOVAs of four variables (growth, deformation score, individual variability of growth, and composite merit) with three factors, regime (R) (two), cross (C) (two; inbred or outbred), and strain (S) (two; Nifi or Israel). N = 511; RXC, RXS, and CXS = interaction terms; df = degrees of freedom, MS = mean square, and P = probability.

		Growth (r^2 = 0.355)		Deformation score (r^2 = 0.398)		Variability of growth (r^2 = 0.084)		Composite merit (r^2 = 0.457)	
Source	df	MS	P	MS	P	MS	P	MS	P
Regime	1	3.862	<0.001	16.840	<0.001	4.901	<0.001	826.308	<0.001
Cross	1	0.122	<0.05	1.819	<0.001	0.012	n.s.	32.419	<0.001
Strain	1	1.464	<0.001	2.691	<0.001	0.434	n.s.	96.317	<0.001
R×C	1	0.013	n.s.	0.068	n.s.	0.002	n.s.	0.001	n.s.
R×S	1	0.008	n.s.	0.517	<0.01	0.478	n.s.	12.129	<0.05
C×S	1	1.410	<0.001	0.237	n.s.	0.450	n.s.	92.651	<0.001
Error	504	0.024		0.065		0.137		2.438	

of inbred groups was dependent on strain. In general, inbreds grew 3.5% less, had 10.42% higher deformation scores, and had 5.88% less individual variability of growth than outbreds. The Nifi strain grew 13.07% less, had 13.32% higher deformation scores, and had 22.40% less individual variability of growth than the Israel strain (Table 3).

Correlation at Individual Level: Within Groups

Regime FE was, on average, a more stressful macroenvironment than was regime CE. Within each regime, every container was a separate environment. This was particularly true for fish that might grow in accordance with position in a social hierarchy, which defines the microenvironment. Consequently, individual response was dependent on each social environment, and in this study each of 48 regime/line/family combinations represented a separate environment. Correlations among growth, deformation score, and individual variability of growth were calculated within each of the 48 combinations, i.e. each container (Table 5).

The underlying distribution of these correlation coefficients was unknown, so that the mean of several coefficients was uninterpretable using parametric statistics. However, we could conservatively assume that under a null hypothesis of zero true correlation, random observed correlation in the positive and negative directions was equally probable. We tested the significance of within-container correlations by testing if the observed frequency of negative and positive correlations that comprised the average differed from an expected ratio of 1:1. Goodness of fit of single classification frequency distributions (Sokal and Rohlf 1981) was used to test the following correlations: (a) deformation score and individual variability of growth, (b) deformation score and growth, and (c) individual variability of growth and growth (Table 6). The G values (adjusted for sample size less than 200) for each relationship (a), (b), and (c) differed significantly from our null hypothesis of a 1:1 ratio. Overall, relationship (a) was positive and (b) and (c) were negative. Therefore, in the same container, individuals which grew rapidly had more stable growth and higher developmental stability than individuals which grew slowly.

Correlation at the Family Level: Among Groups

The only significant correlations were within regime CE in which the correlation between deformation score and growth was significantly negative and the correlation between growth and individuality of growth was significantly positive (Table 7). If fish did not grow at all, as was possible in regime FE, circuli were not laid down (Bilton and Robins 1971a, 1971b), deformed or not. It was possible that regime FE suppressed growth sufficiently that some fish did not grow, the average deformation score was artificially low, and thus the relationship between growth and deformation score was obscured.

Growth at the family level was negatively correlated with growth stability whereas at the individual level, the relationship was positive. We cannot assume that the (macro) environmental differences observed at the family level in a single dietary regime were greater than the (micro) environmental differences experienced by individuals grown together within families. However, the relevance to genetic improvement programs is that families or strains are used to test performance across a range of environments, but individuals do not experience more than one macroenvironment. The result is that the sign of the correlations at the family level apply only when dealing with discrete macroenvironments.

In summary, on average, families which grew faster had a less stable pattern of growth, but within each family, individuals with higher growth rates had a more stable pattern of growth (Fig. 3).

Discussion

This paper presents an operational method of estimating developmental and growth stabilities by measuring scale morphological features. Our results demonstrate how indices of developmental and growth stabilities can be used to characterize fish merit. A composite index combines three criteria of relative merit; the example here combines fast growth with high developmental and growth stabilities. A composite definition of merit extends definitions that are based on growth alone. It is also, in a bioeconomic sense, more realistic and consistent with what is considered to be superior merit in other crops and domesticated animals.

The three traits, growth, developmental stability, and growth stability, may be genetically and environmentally correlated, or they may not be. Each trait has a genetic variance and a heritability and each can be considered separately, from both a genetic and an economic viewpoint. As indices of economic merit, both developmental and growth stabilities have value, as they may ensure viability of broodstock over generations and improve predictability and reduction of risk. However, the actual bioeconomic strategy will depend on the degree of environmental variability in the rearing system (Jinks and Pooni

TABLE 5. Individual level: Pearson product moment correlations within each family (1–4) in each regime (n = average 16 individuals per regime/line/family combination). Correlation coefficients are between the following variables: (a) deformation score and individual variability of growth, (b) deformation score and growth, and (c) growth and individual variability of growth.

	(a)	(b)	(c)
		Regime FE	
Inbred Nifi			
1	−0.328	−0.258	0.604
2	0.425	0.120	−0.445
3	0.172	−0.348	0.534
4	0.079	−0.042	0.599
Outbred Nifi			
1	0.390	−0.633	−0.122
2	0.056	−0.423	−0.426
3	0.212	−0.009	−0.047
4	−0.114	−0.359	0.086
Inbred Israel			
1	0.337	−0.339	−0.272
2	0.138	−0.010	0.177
3	0.168	−0.618	0.016
4	0.687	−0.266	−0.473
Outbred Israel			
1	0.188	−0.102	−0.117
2	0.494	−0.271	0.191
3	−0.072	−0.447	0.316
4	0.340	−0.018	−0.118
Hybrid I × N			
1	0.697	−0.363	−0.259
2	0.221	0.070	0.000
3	0.385	−0.256	−0.188
4	0.079	−0.395	−0.205
Hybrid N × I			
1	−0.137	−0.134	−0.315
2	0.311	0.314	−0.328
3	0.055	0.487	−0.148
4	−0.629	0.374	−0.115
		Regime CE	
Inbred Nifi			
1	0.282	−0.310	−0.078
2	−0.457	−0.172	−0.047
3	−0.257	−0.172	−0.022
4	0.610	−0.354	−0.220
Outbred Nifi			
1	0.350	−0.530	0.243
2	0.043	0.190	−0.009
3	0.249	0.197	−0.050
4	0.318	−0.200	0.346
Inbred Israel			
1	0.004	0.262	0.077
2	0.216	0.162	0.029
3	0.525	0.295	0.261
4	0.515	−0.144	−0.342
Outbred Israel			
1	0.199	−0.298	−0.151
2	0.198	0.550	0.165
3	0.335	0.091	0.174
4	0.107	0.177	−0.042
Hybrid I × N			
1	0.077	−0.032	−0.320
2	0.136	0.187	0.231
3	−0.162	0.342	−0.657
4	0.166	−0.008	−0.270
Hybrid N × I			
1	−0.070	0.004	−0.041
2	−0.353	−0.265	0.721
3	0.195	−0.316	−0.128
4	−0.256	0.427	−0.061

TABLE 6. G-test to determine if the frequency ratio of the signs of observed data of (a), (b), and (c) (see Table 5) were significantly different from the expected ratio of 1:1. $^{***}\chi^2_{0.001(1)} = 10.827$; $^{**}\chi^2_{0.01(1)} = 6.635$, $^{*}\chi^2_{0.05(1)} = 3.841$.

Sign of correlations	Observed	Expected	G (adjusted)
(a) −	11	24	14.72***
+	37	24	
(b) −	32	24	5.38*
+	16	24	
(c) −	31	24	4.14*
+	17	24	

TABLE 7. Family level: Pearson product moment correlation using family means within regime (n = 24 families within each regime; n = 48 when regimes are pooled). Correlation coefficients are between the following variables: (a) deformation score and individual variability of growth, (b) deformation score and growth, and (c) growth and individual variability of growth. *Significant at the 0.05 probability level; **significant at the 0.01 probability level.

	(a)	(b)	(c)
Regime FE	0.105	−0.130	0.369
Regime CE	−0.094	−0.586**	0.470*

1988) and on the extent of genetic correlations among variables. The underlying genetic correlations are important because (1) negative genetic correlations between variables can constrain selection or (2) positive genetic correlations between variables can result in inadvertent selection of an undesirable trait (Rosielle and Hamblin 1981; Lande 1982; Jinks and Pooni 1988).

Developmental stability, as measured by the inverse of deformation scores, reflects a physiological ability to adhere to a genetic plan, relative to other individuals. Animals that have high levels of developmental stability across a range of environments are presumably desirable breeders, other traits being equal. With respect to growth and individual variability of growth, it is not immediately clear whether growth stability across a range of environments is economically or biologically advantageous, when measured at the family level. From an applied or practical standpoint, the possible strategies must also include socioeconomic goals. For example, growth stability is advantageous if it is desired that families be insensitive to uncontrolled fluctuations in the environment. Growth instability may be desired if the goal is to enhance the economic security of small-scale fish farmers by selecting strains that do best in specialized or local environments or respond well to added food (R. W. Doyle, N. L. Shackell, Z. Basiao, S. Uraiwan, and A. Talbot, unpubl. data). Stability is desired if environmental variation is considered to be a stress, such as poor water quality, but may not be desired if environmental variations are considered to be an advantage, such as added food. In agriculture, assessments can vary because better strains are often referred to as "unstable" in stressful environments yet "more responsive" to added inputs (Jinks and Pooni 1988).

From an evolutionary perspective, either negative or positive genetic correlations between growth and individual variability of growth may be adaptive, in fact the dichotomy defines the two possible strategies an organism can adopt in the face of environmental change (reviews in Bradshaw 1965; Schlichting

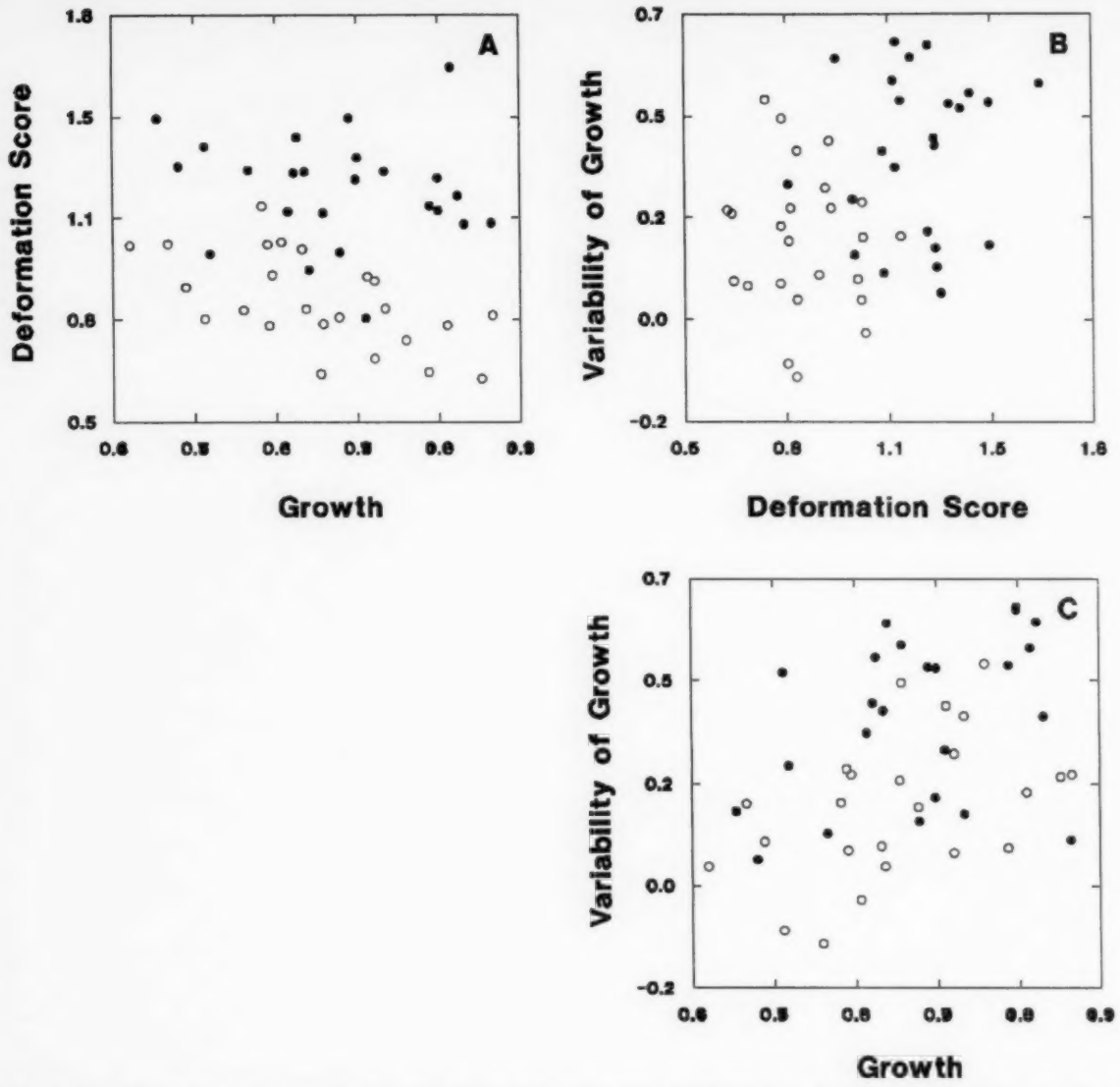

FIG. 3. Relationships among (A) deformation score (log number of deformed circuli) and growth (% log mm/d), (B) individual variability of growth (log of variance of circulus spacing) and deformation score, and (C) individual variability of growth and growth. Solid symbols = variable and less nutritious regime FE; open symbols = constant and more nutritious regime CE (N = average 16 per regime family combination).

1986). Via (1987) maintained that genetic variation in response to the environment allows the evolution of phenotypic plasticity (one genotype resulting in adapted phenotypes in response to environment) which is itself an adaptation. On the one hand, if the genetic correlation between growth rate and growth stability is negative, then the genetic correlation may reflect the possibly adaptive ability to stabilize growth in a heterogeneous environment. On the other hand, if growth and growth stability are positively correlated (e.g. in *Pinus attenuata*; Strauss 1987), then sensitivity to the environment reflects the possibly adaptive ability to be an opportunist by increasing growth in favorable environments (Strauss 1987). If fish breeders select for phenotypic stability across environments when the correlation between growth and variability of growth is positive, there is a risk of losing a valuable adaptation, that is the ability to respond optimally and maximize growth when conditions permit (Via 1987).

Our results show that growth is positively correlated with growth stability at the individual level (among social microenvironments), yet negatively correlated at the family level (among environmental macroenvironments). In addition, the family correlation between growth and individual variability of growth is nonsignificant in the restrictive regime FE, yet significantly positive in the nonrestrictive regime CE. We favor the explanation that, at the individual level, each fish is reacting to other fish. Therefore, the growth of small submissive fish is sensitive to that of large fish, the access to food of small fish will be more variable, and also their growth will be more variable. However, in each family, the dominant fish are capable of enhancing their growth rates when conditions permit. The

ability to do so differs among families. That is, some families include individuals that can respond to good environments and others do not. Our results suggest an opportunistic response at the family level to the nutritional environment. Hypothetically, within each family, fast growers will maximize growth until they are limited by their physiology or the environment, and then they will compensate by slowing growth. The result is a more variable growth pattern among fast growers.

A positive correlation between growth and individual variability of growth at the family level is of importance to genetic improvement programs because families or strains are used to compare across environments. That is, individuals are not replicated across environment. Therefore, the family or strain correlations should be considered when developing a bioeconomic plan. In any event, fish breeders should choose fish based on the underlying genetic correlations among growth and developmental and growth stabilities that are established in the future rearing environment.

We present a method to evaluate tilapia merit. Composite indices of merit have three potential applications. The first is as an indicator of environmental stress, where the index could be used to detect sublethal stress of broodstock over time or between aquacultural systems. The source of stress can be inferred by coupling the results with technical information about a particular system (e.g. poorer quality of food, change in pH, introduction of competitor species). The second application is as an indicator of genetic stress, e.g. inbreeding to compare populations in a single environment. The third application is as a selection index to compare individuals within environment × population combinations.

An immediate application is to compare levels of abnormal scale morphology among fish in different aquaculture systems to diagnose stressful environments. The eventual use of the scale morphological technique is in a selection experiment, designed to identify superior genotypes on artisanal farm systems. Whatever selection procedure is used, it would be wise to enhance and exploit the natural biology of tilapia by selecting for a composite merit of growth and stress resistance. A composite index incorporates three important economic traits and may differentiate individual, family, and population overall merit more accurately than any one trait alone.

Acknowledgements

We are grateful to Ruel Eguia, Zubaida Basiao, Boening Antazo, and Jerry Jacusalem for their helpful ideas and technical assistance. The work was supported by the International Development Research Centre (IDRC; Canada) and by postgraduate IDRC and Natural Sciences and Engineering Research Council of Canada scholarships to the senior author.

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Riverine and Estuarine Migratory Behavior of Coho Salmon (*Oncorhynchus kisutch*) Smolts

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Moser, M. L., A. F. Olson, and T. P. Quinn. 1991. Riverine and estuarine migratory behavior of coho salmon (*Oncorhynchus kisutch*) smolts. Can. J. Fish. Aquat. Sci. 48: 1670–1678.

Using radio and ultrasonic transmitters, we tracked coho salmon (*Oncorhynchus kisutch*) smolts in the Chehalis River and Grays Harbor estuary, Washington, to document patterns of seaward migration. In spite of dramatic differences in the two habitats, the fish behaved similarly in both systems. Migratory progress was saltatory, characterized by movement in the direction of the current and extended periods of holding in areas of low current velocity. Consequently, smolts were displaced rapidly downstream by swift, unidirectional river currents but were retained in the estuary by relatively low-velocity, reversing tidal currents. Smolts did not use tidal currents to move seaward and appeared to swim most rapidly against strong ebbing currents. Estimated mean total metabolic cost incurred by migrating smolts was 0.027 cal·s^{-1}·smolt^{-1} (1 cal = 4.184 J). Estimated daily caloric expenditure by smolts in 1988 was twice that of smolts tracked in 1989, apparently due to faster water velocities in 1988. The observation that coho salmon migration through estuaries is slower than riverine migration suggests that a period of estuarine residence may be necessary for them to adjust their osmoregulatory capability, orient for their return migration, feed, or reduce their vulnerability to predators.

À l'aide d'émetteurs radio et à ultrasons, nous avons suivi dans la rivière Chehalis et l'estuaire de Grays Harbor (État de Washington) des smolts de saumon coho (*Oncorhynchus kisutch*) pour étudier les modes d'avalaison. Malgré des différences frappantes entre les deux habitats, les poissons se comportaient de la même façon dans les deux systèmes. Le mouvement migratoire était discontinu, et caractérisé par un déplacement dans le sens du courant et de longues périodes d'attente dans des zones où la vitesse du courant est lente. Les smolts étaient donc emportés rapidement vers l'aval par les courants rapides unidirectionnels dans la rivière mais étaient retenus dans l'estuaire par les courants de reflux à vitesse relativement faible. Les smolts n'utilisaient pas les courants de marée pour descendre en mer et semblaient nager à une vitesse maximale contre les forts courants de reflux. On estime la dépense métabolique totale moyenne des smolts en migration à 0,027 cal·s^{-1}·individu^{-1} (1 cal = 4,184 J). La dépense calorique quotidienne estimée pour les smolts en 1988 était le double de celle des smolts suivis en 1989, ce qui semble dû à la plus grande vitesse des eaux en 1988. Le fait que la migration des cohos dans les estuaires soit plus lente que la migration en rivière indique qu'une période de résidence estuarienne peut leur être nécessaire pour ajuster leur capacité d'osmorégulation, s'orienter en vue de la migration de retour, se nourrir ou réduire leur vulnérabilité aux prédateurs.

Received May 25, 1990
Accepted March 15, 1991
(JA586)

Reçu le 25 mai 1990
Accepté le 15 mars 1991

During migrations from freshwater to the open ocean, juvenile salmonids encounter dramatic changes in their physical environment while undergoing physiological and behavioral changes associated with smolt transformation. Based on work with Atlantic salmon (*Salmo salar*), Tytler et al. (1978) hypothesized that this transition period may be a survival bottleneck as smolts incur higher predation risk, cope with osmoregulatory adaptation, and assimilate information needed to successfully return as adults. Seaward migration routes and rates of travel are probably determined by the interaction of these factors and by the precision of smolt orientation.

Orientation mechanisms used by migrating salmon smolts apparently differ among species and habitats. Sockeye salmon (*Oncorhynchus nerka*) smolts use compass orientation (Groot 1965; Quinn and Brannon 1982) whereas Atlantic salmon smolts orient primarily to currents (Fried et al. 1978; Tytler et al. 1978; Thorpe et al. 1981). The development of miniaturized transmitters permitted us to document for the first time the estuarine migratory behavior of individual Pacific salmon smolts. We tracked coho salmon (*Oncorhynchus kisutch*) smolts in Grays Harbor estuary, Washington, to determine migration routes and and rates of travel, and we documented the riverine phase of migration of these fish by radio tracking. By comparing migratory behavior of coho salmon in the riverine and estuarine systems, we tried to identify general behavior patterns and the environmental cues most important to smolt orientation.

Predictions of smolt growth, and consequent fitness, require accurate estimates of migratory costs (Wissmar and Simenstad 1988). These costs are determined by both smolt swimming speeds and duration of activity. We estimated smolt swimming speeds from ground speed and current velocity vectors in the estuary and used these data to estimate energetic costs (calories per second) incurred by smolts during periods of activity. By selectively riding ebbing currents and holding during flooding currents, coho salmon smolts could speed their seaward migra-

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tion at low metabolic cost (Arnold 1981). To determine whether smolts use this mechanism, we compared estimated smolt swimming speeds and orientation to the current during both ebb and flood tides. These data and observations of the percent time spent actively migrating were used to estimate total metabolic expenditures for the estuarine phase of smolt migrations.

Materials and Methods

Study Site and Tagging Protocol

Smolts tagged with radio transmitters were tracked in the Chehalis River, Washington, from river km 67 to 29 in 1989 (Fig. 1). Smolts tagged with ultrasonic transmitters were tracked from the lower Chehalis River into Grays Harbor estuary in 1988 and 1989 (Fig. 1). Tidal influence generally did not extend upstream of km 22 in the Chehalis River. The upper extent of the salt wedge was encountered in the region from km 3 to 14, depending on tide stage and river discharge. River depth ranged from < 2 m at km 67 to > 10 m in the dredged channel of the lower estuary. A Secchi disc was typically visible through the entire water column in the river above km 24, but often could not be seen below 1 m in the estuary.

In both years, the study's duration (April–June) bracketed the May peak of coho salmon smolt migration. Wild smolts were collected from a weir in Scatter Creek, a tributary of the Chehalis River, and held in 1.5 × 1.5 × 0.5 m, 4-mm-mesh holding pens, located within 1.0 km of release sites. Smolts were held no longer than 3 d prior to radio tagging and tracking and no longer than 6 d for ultrasonic tracking. Wooden baffles were used to reduce water flow through the pens, protecting smolts from rapid current flows. For radio tagging, we anaesthetized each smolt with MS-222 (50 mg·L^{-1}, 2–4 min) and gently pushed a transmitter down the esophagus with a paper straw until the transmitter was no longer visible. For ultrasonic tagging, we used a smooth wooden rod to insert each transmitter. Tagged smolts were allowed to recover for at least 30 min in a 45-L cooler and then returned to the holding pens for at least 24 h.

Riverine Tagging and Tracking

For riverine tracking, smolts were tagged 1–2 d before release with radio transmitters developed by the National Marine Fisheries Service (Stuehrenberg et al. 1986). Radio transmitters were 26 × 9 × 6 mm flattened cylinders with a 127-mm flexible whip antenna and weighed 2.9 g in air (0.8 g in water). A 4 × 7 mm piece of cellulose sponge was attached to the transmitters with cotton thread to minimize tag regurgitation. Only smolts >160 mm fork length (mean = 169 mm) were used, so that transmitters would not alter smolt behavior or swimming performance (Moser et al. 1989). Each transmitter had a unique combination of frequency and pulse period, allowing us to identify individual smolts. Battery life was 5–7 d.

The first four smolts were released at Chehalis River km 83, but subsequent reductions in flow forced us to move the release site downstream to km 67 (Fig. 1). Transmitter signals were detected from a jet-propelled boat using a directional loop antenna and receiver (Smith-Root RF-40). Smolt locations were recorded at approximately 20-min intervals for up to 10 h following release of each fish. Smolt ground speed was estimated as the distance travelled between observations divided by the time interval between observations.

Two remote receiving stations, located at km 53 and 29, also monitored smolt passage (Fig. 1). These units, developed by the National Marine Fisheries Service, continuously scanned for transmitter signals. When tagged smolts passed by, the indi-

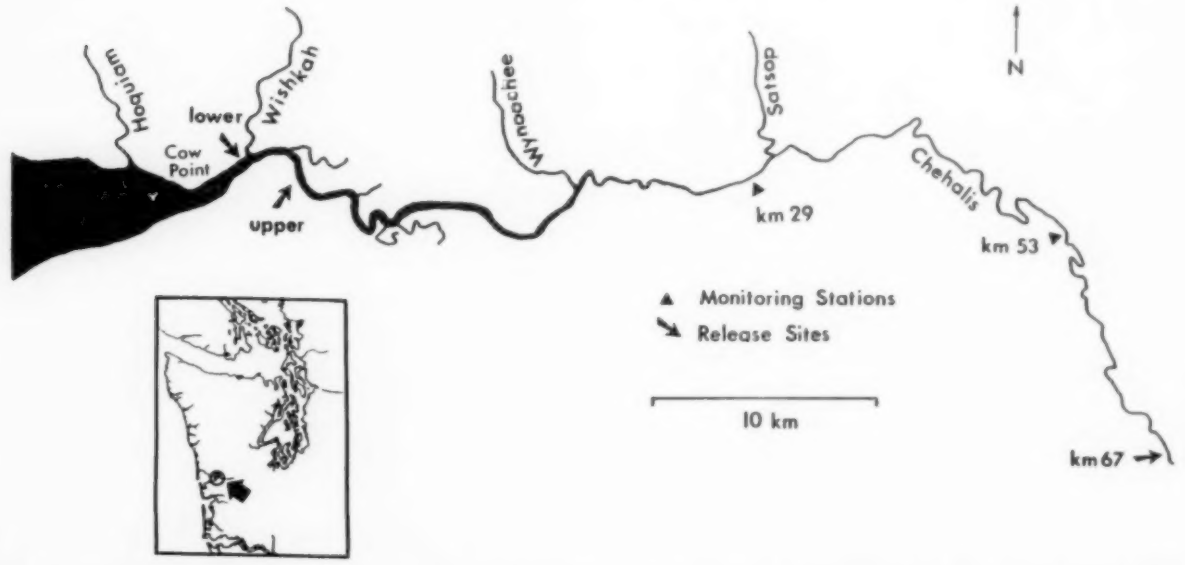

FIG. 1. Coho salmon smolt tracking study area in the Chehalis River and Grays Harbor estuary, Washington. The upper riverine release site and Independence Creek (both not shown) were 16 km upstream of the lower riverine release site (arrow at km 67). The arrow marking the lower estuarine site also indicates km 0.

TABLE 1. Migration rates of six coho salmon smolts tracked in the Chehalis River, Washington, calculated on the basis of continuous ground speed measurements and as gross rates. Gross rate of travel was the total distance travelled (data from remote stations) divided by the total time tracked (h).

Smolt No.	Fork length, mm	Hours tracked continuously, no. of obs.	Ground speed, $km \cdot h^{-1}$ ($cm \cdot s^{-1}$)	Total hours tracked	Gross rate, $km \cdot h^{-1}$
5	185	3.5 (14)	3.2 (88.9)	24.3	1.6
7	163	5.5 (12)	2.4 (66.7)	6.5	0.8
9	167	7.0 (14)	2.9 (80.6)	26.0	1.4
10	192	3.5 (15)	4.3 (119.4)	21.5	1.7
14	165	6.5 (13)	2.5 (69.4)	71.5	0.5
18	165	3.5 (12)	3.4 (94.4)	27.5	1.4
$\bar{x}$	173	4.9 (13)	3.1 (86.1)	29.6	1.2

vidual fish code, time, and date were recorded. Estimates of total distance travelled downstream and gross travel rate (total distance divided by total track time) were obtained from these data. Effects of smolt size, release date, and water temperature on gross distance and rate of travel were examined by use of the Kendall test (Zar 1984).

Surface water temperature and current velocity (AquaMeter No. 660) were recorded when possible during periods of smolt movement. However, the high variability of local current velocity and the low resolution of fish locations (±30 m) prevented us from determining the currents actually experienced by the smolts. Vertical current profiles were also recorded at five stations located at 8-km intervals from km 83 to 53. These measurements were taken three times during the course of the study to document trends in water flow.

Estuarine Tagging and Tracking

For estuarine tracking, coho salmon smolts were tagged 1–3 d before release with ultrasonic transmitters (Vemco V2B-1L). Each transmitter had a unique combination of frequency and pulse rate to permit identification of individual smolts. The transmitters were cylindrical, 33 × 9 mm, and weighed 4.6 g in air (2.5 g in water). Battery life was rated at 6 d, but we detected signals from some transmitters up to 12 d after activation. Mean smolt fork length was 187 mm in 1988 and 168 mm in 1989 (Table 1).

Two release sites in midchannel of the Chehalis River estuary were chosen: upper and lower (Fig. 1). In 1988, the first five smolts were released at the upper site and the remaining 10 at the lower one. In 1989, all eight smolts were released at the lower site. A directional hydrophone and receiver (Vemco Ltd.) were used to detect transmitter signals from a boat, and smolt locations were determined (±50 m) using a combination of triangulation and signal strength. Location and water depth were recorded every 10 min for up to 16 h following release of each smolt. A three-point running average of ground speed observations was calculated to reduce over- or underestimates of speed caused by lack of precision in locating smolts. The percent time each smolt spent holding position (%H) was defined as

$$\%H = 100 \cdot t_h \cdot t_t^{-1}$$

where t_h = the amount of time a smolt spent in one location for at least 1 h and t_t = the total time the smolt was tracked. The Mann–Whitney test (Zar 1984) was used to compare mean ground speed and percent holding time in both study years.

We observed coho salmon smolts swimming near the surface and assumed that they remained near the surface throughout their migration, as observed in studies of Atlantic salmon smolts (Fried et al. 1978; LaBar et al. 1978). Therefore, we measured current velocity and direction (AquaMeter No. 660) and salinity and temperature (Hydrolab Scout) at a depth of 1 m every 30 min during continuous tracking. Vertical profiles of salinity and temperature were also taken at irregular intervals. Surface salinity distributions measured during moving and holding periods for each smolt were compared using Kolmogorov–Smirnov tests (Zar 1984). We used the Mann–Whitney test to make between-year comparisons of mean estuarine current velocities measured during periods of smolt movement from April 20 to May 2 in 1988 and from May 15 to 24 in 1989. This test was also used to compare mean ebbing and flooding current velocities during periods of smolt movement in both years. Correlation between ground speed and the current velocities during periods of smolt movement was examined using the Kendall test.

Current speeds were subtracted from smolt ground speeds to obtain swimming speed estimates. A type of repeated measures analysis (with unbalanced data) was performed to examine the effects of year (fixed effect), current direction (fixed effect), smolt orientation to the current (fixed effect), and current velocity (covariate) on estimated smolt swimming speed. To test these factors, an error based on smolts as replicates was calculated. This error was significant ($P = 0.05$) relative to the error based on variation within observations made on the same smolt. The year effect was tested against the interaction between the orientation and current direction effects with fish within years. That is, main effects and interactions for fixed effects were tested against errors based on individual smolts as replicates.

Swimming speed estimates, u (body lengths per second), were used to calculate total metabolic expenditure, T (calories per second), during periods of smolt movement using the following relationship:

$$T = a\, w\, Q_{ox} Y_u$$

where a = a conversion factor to convert joules to calories (i.e. 0.239 $cal \cdot J^{-1}$), w = smolt wet weight (grams) obtained from a length–weight conversion developed for Chehalis River coho smolts ($w = 10^{-4.7}$ (millimetres fork length)$^{2.88}$; K. Fresh, Washington Department of Fisheries, pers. comm.), Q_{ox} = oxycalorific coefficient to convert milligrams oxygen consumed by smolts to joules of energy lost as heat (i.e. 14.0 $J \cdot mg\ O_2^{-1}$, and Y_u = oxygen consumed (milligrams oxygen per gram per second) by fish moving at swimming speed u

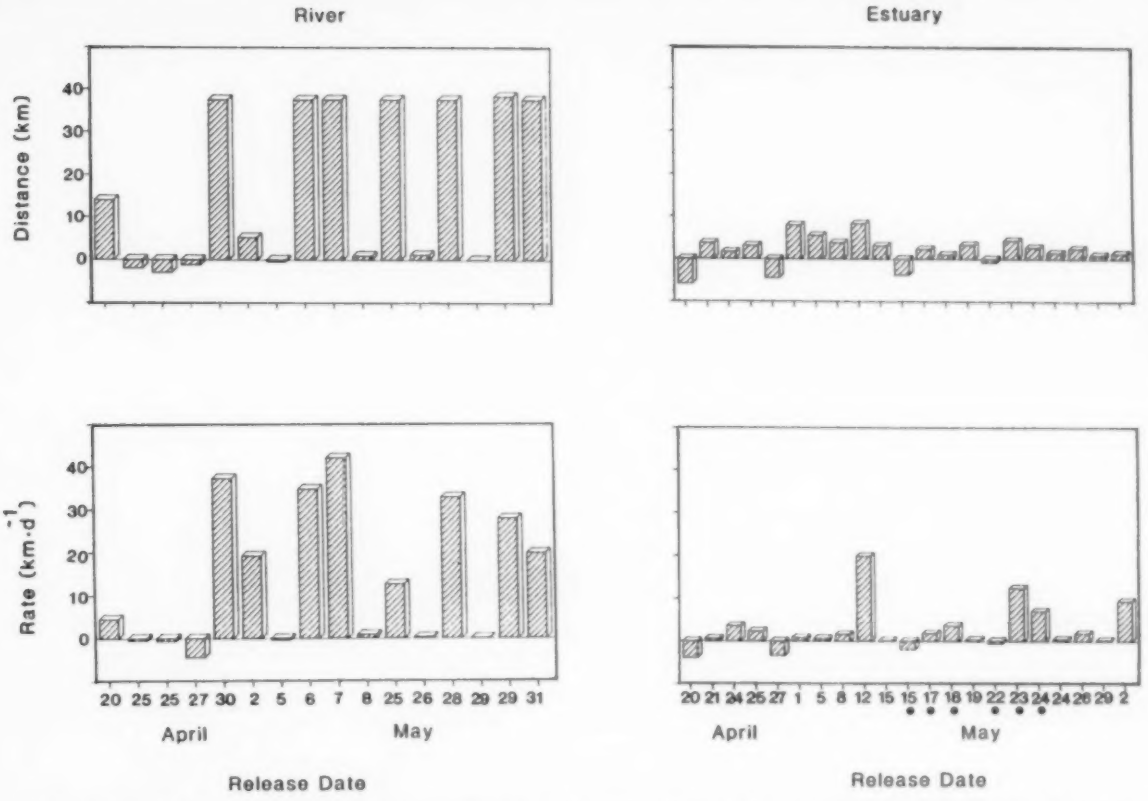

FIG. 2. Gross distance travelled downstream (upper panels) and rate of travel (lower panels) for each coho salmon smolts tracked in the Chehalis River (1989) and in Grays Harbor estuary (1988–89), Washington. Hatched bars indicate results for each individual smolt (including those presented in Table 1). The date each smolt was released is given below each bar, with dots indicating estuarine releases made in 1989.

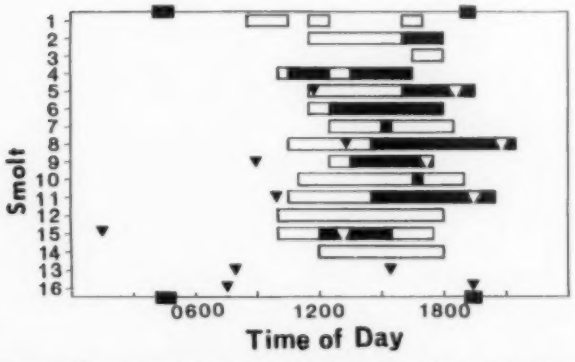

FIG. 3. Observed periods of coho salmon smolt activity in the Chehalis River, Washington. Open bars represent continuous tracking of individual smolts, and darkened areas indicate periods of smolt movement. Triangles show times when smolts passed the remote monitoring stations. The range of sunrise and sunset times during the study (April 20 to May 31, 1989) are indicated by solid boxes on the time axis.

(body lengths per second). Because an oxygen consumption – swimming speed relationship for coho salmon smolts was not available for the fish sizes and water temperatures of our study, we used the relationship Y_u (mg $O_2 \cdot g^{-1} \cdot s^{-1}$) $= 2.8 \times 10^{(-5.15 + 0.27u)}$ developed by Brett (1964) for sockeye salmon.

Since

$$T = T_m + T_s$$

where T_m = the metabolic costs of swimming (calories per second) and T_s = standard metabolic costs (i.e. T at $u = 0$), we estimated the percentage of total metabolic costs used for swimming ($\%T_m$) by

$$\%T_m = 100(T - T_s) \cdot T^{-1}.$$

Total metabolic costs during periods of active migration (T) and $\%T_m$ expended in ebbing and flooding currents were estimated for each smolt. Based on our observations, we assumed that smolts were equally active during flooding and ebbing tides and that during holding periods, $u = 0$. Therefore, total daily metabolic costs, T_d (calories per day), incurred by smolts in the estuary were approximated by

$$T_d = 100(\%HT_s + 0.5\%M(T_{ebb} + T_{flood}))$$

where $\%H$ = percent time spent holding, $\%M$ = percent time spent moving (i.e. $100 - \%H$), $T_{ebb} = T$ during ebbing currents, and $T_{flood} = T$ during flooding currents.

Tagged smolts were often relocated on subsequent days during midchannel transects of the river with the hydrophone. Gross downstream travel distance was defined as the distance from the release site to the position recorded when the smolt

was last located. Gross travel rate was gross distance divided by the total time each smolt was tracked. Gross travel distance and rate were only calculated for smolts tracked longer than 1 h. We tested for between-year differences in mean gross travel distance and in gross travel rate with the Mann–Whitney test and for effects of smolt size or release date with the Kendall test.

Results

Riverine Tracking

Of the 19 smolts tagged and released in 1989, we were unable to track three owing to transmitter malfunction. Of the remaining 16 smolts, three swam up Independence Creek, a tributary of the Chehalis River near the upper release site, and were not subsequently located in the river. Four smolts held position within 1.5 km of the release site until their transmitters ceased functioning. Three smolts migrated downstream during periods when we were not continuously monitoring them. The remaining six smolts moved downstream during periods of continuous monitoring at a mean ground speed of 3.1 $km \cdot h^{-1}$ (86.1 $cm \cdot s^{-1}$) (Table 1). Observed mean current speed during periods of fish movement was 3.3 $km \cdot h^{-1}$ (91.7 $cm \cdot s^{-1}$). However, it was impossible to determine whether smolts were actually experiencing the current velocities measured. Therefore, we did not attempt to calculate fish swimming speeds from the riverine tracks.

Of the nine smolts which moved downstream, seven were detected by both remote receiving stations as they moved out of the study area. Consequently, gross downstream travel distance for these fish was the same, 38 km (Fig. 2). Gross downstream travel distance and travel rate were not correlated with smolt size ($P > 0.05$), but travel distance was positively correlated with the date of release ($P < 0.05$; Fig. 2). However, when smolts released during April 20–27 at the upper site were removed, this trend was no longer significant ($P > 0.05$), since three of the four smolts released at the upper site swam upstream (Fig. 2). Release date did not affect gross rate of travel ($P > 0.05$; Fig. 2). Mean daily water temperature varied from 10.5 to 17.5°C and was highest during the middle of the study period (smolt releases during April 30 to May 8). Distance travelled was not correlated with water temperature, but travel rate increased with increasing temperature ($P < 0.001$). Mean current velocities decreased from 60 $cm \cdot s^{-1}$ at the beginning of the study period to 40 $cm \cdot s^{-1}$ at its end. Daily flow data collected by the U.S. Geological Service at km 53 also indicated a decrease in river discharge from April 10 (170 $m^3 \cdot s^{-1}$) to May 28 (37 $m^3 \cdot s^{-1}$) 1989.

Mean ground speeds of fish tracked continuously (3.1 $km \cdot h^{-1}$, $n = 6$) were two to five times faster than mean gross travel rates determined from monitoring stations for the same individuals (1.2 $km \cdot h^{-1}$) (Table 1). We observed extended periods of holding by tagged smolts in river spurs and backwater areas where current velocities were very low. This behavior accounts for the discrepancy between ground speed and gross rate calculations. We did not continuously track smolts after dark as a safety precaution; hence, we were unable to establish diel activity patterns. However, fish generally passed the

TABLE 2. Migration activity of coho salmon smolts tracked in Grays Harbor, Washington, including the percent time each smolt held position for at least 1 h and the percent time each smolt went in the direction of the current. The ratio of gross to net distance travelled (for smolts tracked during both tides) indicates movement back and forth with reversing currents.

Year	Smolt No.	Fork length, mm	Time tracked, h	% time moving with current	Gross to net distance	% time holding
1988	2	193	15.3	100	10.6	20.3
	3	173	127.0	100	3.1	62.5
	4	169	32.5	100	9.8	71.7
	5	179	11.0	100	1.8	72.7
	6	163	31.5	45	1.7	75.2
	7	198	183.3	78	1.3	96.6
	8	192	134.0	100	3.4	94.2
	9	170	57.8	100	3.5	90.8
	10	173	10.0	100	1.4	15.0
	11	171	23.8	100	2.1	58.0
	12	183	285.3	100	1.2	99.5
	13	215	17.3	100	2.3	85.6
	14	210	31.5	100	1.3	89.8
	15	209	68.3	86	2.2	95.9
	16	212	3.3	100	—	90.9
	$\bar{x}$	187	68.7	94	3.3	74.6
1989	1	160	1.0	100	—	—
	2	183	12.2	62	3.2	27.0
	3	182	4.0	100	—	62.5
	4	160	7.0	100	4.9	31.4
	5	172	8.5	100	1.2	52.9
	6	159	1.0	100	—	—
	7	160	8.5	100	3.3	78.8
	8	166	9.7	92	3.5	27.8
	$\bar{x}$	168	6.5	94	3.2	46.7

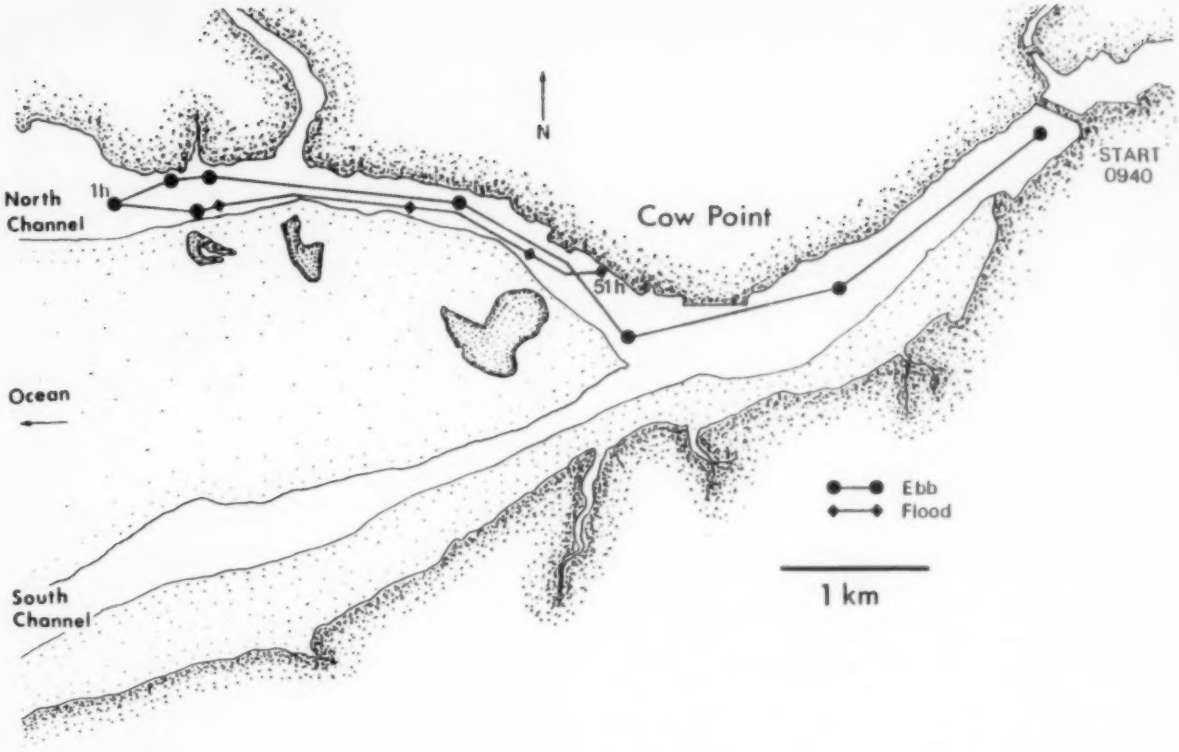

FIG. 4. Locations of coho salmon smolt No. 9 (1988) at 30-min intervals during ebbing (circles) and flooding (squares) tides in Grays Harbor, Washington. Lengths of time spent holding position are shown at the holding sites.

continuous monitoring stations between sunrise and sunset (12 of 14 detections; Fig. 3), suggesting that smolts were most active during daylight.

Estuarine Tracking

Fifteen smolts were tracked in 1988 and eight smolts in 1989 (Table 2). Ten smolts in 1988 and three in 1989 were tracked beyond the junction of North and South channels in Grays Harbor. Six of the 10 smolts in 1988 migrated seaward via the North Channel whereas the remaining smolts used the South Channel. In 1989, one smolt entered the South Channel and the other two moved in and out of both channels.

Smolt ground speeds generally followed tidal current directions, as indicated by the track map of a representative smolt (Fig. 4). Ground speeds during periods of movement for 12 of 15 smolts in 1988 and seven of eight smolts in 1989 were in the same direction as the current more than 90% of the time (Table 2). Consequently, mean ratios of gross to net distance travelled by smolts tracked during both ebb and flood tides were high (3.3 in 1988, 3.2 in 1989; Table 2). Mean gross distance travelled by smolts in 1988 (2.4 km) was not significantly different ($P > 0.05$) from that in 1989 (1.0 km). Similarly, mean gross travel rate in 1988 (2.3 km·d^{-1}) was not significantly different ($P > 0.05$) from that in 1989 (3.7 km·d^{-1}). Neither release date nor smolt size was correlated with distance or rate of travel ($P > 0.05$).

Mean ground speed in both years was greater than gross travel rate because smolts spent a large amount of time holding in areas of low current velocity, particularly around Cow Point (Table 2; Fig. 4). Percent time spent holding by smolts in 1988 (74.6%) was greater than in 1989 (46.7%) (Table 2). Observed residence time in the upper estuary in 1988 averaged about 3 d (69 h), although two smolts (Nos. 6 and 12) stayed near Cow Point for over a week (Table 2). In 1989, mean observed residence time was much shorter (about 1 d).

Water temperature at 1 m ranged from 10 to 14°C during the 1988 study and from 14 to 15°C in 1989 but did not vary by more than 1°C during continuous tracking of individual smolts. In both years, vertical temperature stratification did not exceed 1°C. Of the smolts tracked to the confluence of North and South channels, 70% in 1988 and 100% in 1989 remained at Cow Point over at least one tidal cycle. Surface salinities at Cow Point varied by as much as 12‰ over the course of a tidal cycle. Similarly, salinity measurements taken during continuous tracking indicated that smolts were exposed to salinities ranging from 0 to 20‰. In both years, surface salinity gradients from the release site to Cow Point exceeded 10‰. In 1988, salinity varied as much as 21‰ from surface to bottom at Cow Point but was less vertically stratified in 1989 (not exceeding 11‰). Surface salinity distributions when smolts were moving and when smolts were holding did not differ ($P > 0.05$), suggesting that the smolts did not move to avoid changing salinities.

TABLE 3. Mean current velocity and mean estimated swimming speeds (BL = body lengths) of ultrasonically tracked coho salmon smolts during flood and ebb tides (n = number of observations for each smolt). Total energetic costs incurred while migrating (T) and the percentage of these costs expanded for swimming ($\%T_m$) are also given for each tide.

		Flood			Ebb		
Year	Smolt No.	Mean current velocity, $cm \cdot s^{-1}$	Mean swimming speed, $BL \cdot s^{-1}$ (n)	T, $cal \cdot s^{-1} \times 10^{-2}$ ($\%T_m$)	Mean current velocity, $cm \cdot s^{-1}$	Mean swimming speed, $BL \cdot s^{-1}$ (n)	T, $cal \cdot s^{-1} \times 10^{-2}$ ($\%T_m$)
1988	2	40.1	0.9 (9)	0.88 (43)	74.3	1.7 (8)	1.46 (65)
	3	37.8	0.4 (5)	0.47 (23)	118.1	4.7 (2)	6.85 (83)
	4	—	—	—	75.9	3.3 (12)	2.68 (87)
	5	—	—	—	100.7	4.0 (4)	4.89 (92)
	6	22.5	0.6 (2)	0.45 (32)	113.0	8.4 (4)	57.69 (99)
	7	31.4	2.6 (3)	2.74 (80)	36.1	1.4 (1)	1.30 (58)
	8	17.1	0.4 (6)	0.64 (22)	30.1	1.1 (6)	0.98 (50)
	9	27.8	1.4 (4)	0.84 (58)	61.1	2.1 (7)	1.30 (73)
	10	55.5	2.0 (2)	1.28 (71)	55.2	2.3 (7)	1.54 (76)
	11	72.2	1.8 (8)	1.09 (67)	88.6	3.4 (8)	2.95 (88)
	12	—	—	—	88.0	1.4 (3)	1.03 (58)
	13	—	—	—	58.3	2.2 (3)	2.71 (74)
	14	—	—	—	62.2	0.3 (1)	0.78 (17)
	15	48.6	1.2 (4)	1.34 (53)	57.4	1.1 (3)	1.26 (50)
	16	—	—	—	102.8	0.4 (1)	0.85 (22)
	$\bar{x}$	39.2	1.3 (5)	1.08 (50)	74.8	2.5 (5)	5.88 (66)
1989	1	—	—	—	8.3	0.9 (1)	0.52 (43)
	2	27.8	1.3 (1)	0.97 (56)	46.7	1.8 (10)	1.33 (68)
	3	44.4	1.5 (2)	1.08 (61)	—	—	—
	4	26.8	0.4 (3)	0.38 (22)	24.1	1.3 (3)	0.66 (58)
	5	8.3	0.2 (1)	0.41 (12)	73.6	2.5 (2)	1.72 (79)
	6	36.1	0.8 (1)	0.48 (39)	—	—	—
	7	44.4	1.6 (1)	0.80 (63)	—	—	—
	8	10.1	1.2 (8)	0.69 (53)	48.2	1.8 (3)	1.00 (67)
	$\bar{x}$	28.3	1.0 (2)	0.69 (44)	40.2	1.7 (4)	1.05 (63)

Current velocity measurements were consistent with published tidal current velocity projections (1988–89 Tidal Current Tables, National Oceanic and Atmospheric Administration). Mean current speed during periods of smolt movement in 1988 was much greater than in 1989, and ebbing current velocities exceeded flooding current velocities in both years (Table 3). Mean smolt ground speed was significantly higher in 1988 (42.9 $cm \cdot s^{-1}$) than in 1989 (29.9 $cm \cdot s^{-1}$) ($P < 0.05$) and was positively correlated with current speed in both years ($P < 0.05$).

Repeated measures testing indicated that estimated smolt swimming speed varied significantly among individuals ($P < 0.05$) but not between years ($P > 0.05$). Estimated smolt swimming speed was not affected by current direction ($P > 0.05$) but was strongly influenced by current velocity and the smolts' orientation to the current. In general, smolts swam rapidly against fast currents but swam slowly both with and against slow currents (Fig. 5), resulting in a significant interaction of current direction and smolt orientation effects ($P < 0.05$). Other interactions were not significant.

Mean total energetic expenditure of the smolts during periods of movement (T) was 0.027 $cal \cdot s^{-1} \cdot smolt^{-1}$ (standard deviation 0.058 $cal \cdot s^{-1}$). Because ebbing current velocities exceeded flooding current velocities, smolts swam faster and expended more energy (T) during ebbing tides in both years (Table 3). Reduced current velocities in 1989, relative to those measured in 1988, resulted in lower energetic expenditures by smolts in 1989 (Table 3). Based on T estimates for ebb and flood tides and the amount of time the fish spent holding, we estimated that the mean daily energy consumption (T_d) by smolts in 1988 was 1398 $cal \cdot smolt^{-1}$ or 21.2 $cal \cdot g^{-1}$. In 1989, mean T_d was 605 $cal \cdot smolt^{-1}$ or 11.3 $cal \cdot g^{-1}$.

Discussion

Migratory behavior of coho salmon smolts was similar in the upper Chehalis River and in Grays Harbor estuary, in spite of differences in water turbidity, depth, salinity, and flow between the two habitats. Smolt ground speeds were in the same direction as the current in both areas and were comparable with ambient current velocities. Consequently, smolts moved rapidly downstream in the river, which was characterized by high velocity, unidirectional currents, but moved back and forth in the estuary's lower velocity, reversing tidal currents. Migratory behavior was apparently unaffected by changes in salinity. Moreover, in both the river and estuary, downstream movement of coho salmon smolts was interspersed with extended periods of holding in regions of low water velocity. Similar behavior patterns have been observed during tracking studies of Atlantic salmon smolts, (Fried et al. 1978; LaBar et al. 1978; Tytler et al. 1978; Thorpe et al. 1981).

Smolt holding behavior was most common in the region around Cow Point (Fig. 4). Beach seine sampling in Grays Harbor has also indicated that coho salmon smolts congregate at this site (Simenstad and Eggers 1981). Cow Point is located at the junction of the two major channels in Grays Harbor and has many large docks and numerous pilings. Consequently, current

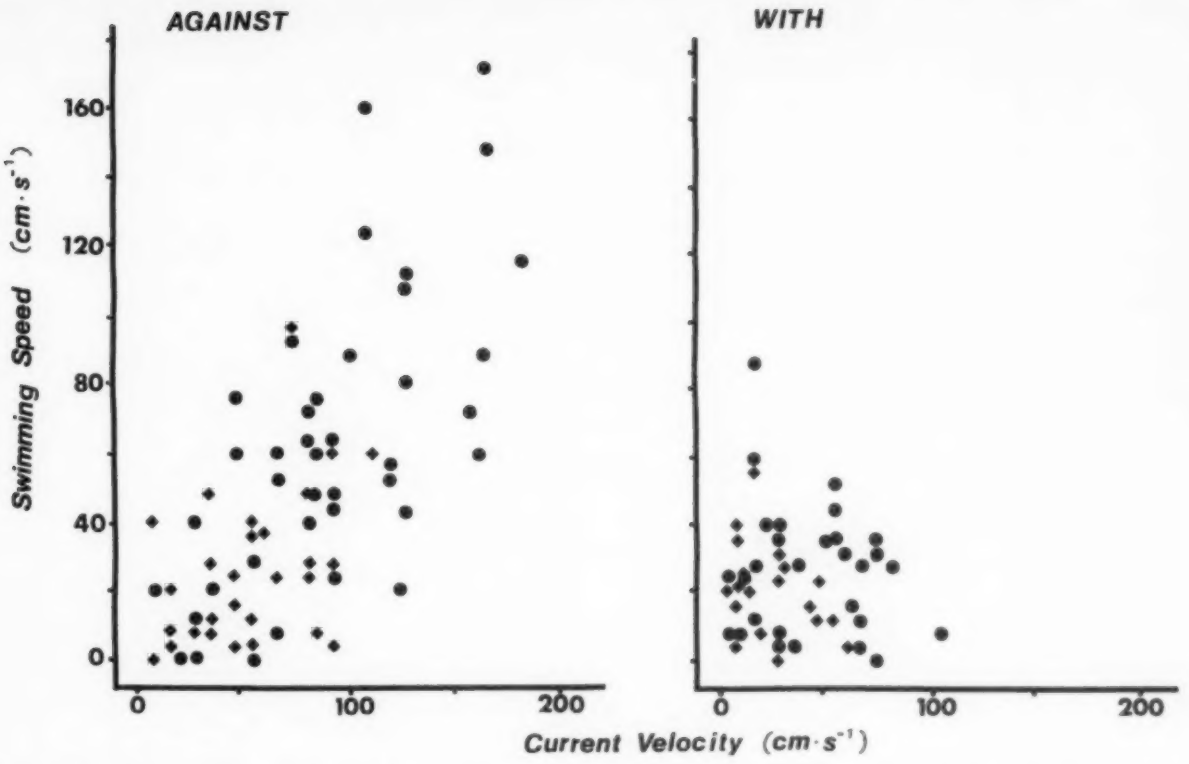

FIG. 5. Estimated coho salmon smolt swimming speeds in response to current velocity when swimming against and with the current during ebbing (circles) and flooding (squares) tides in Grays Harbor, Washington.

flow in this area is complicated by eddies and regions of slack water, which may have served to accumulate smolts. We speculate that prior to development, great quantities of logs and stumps in Grays Harbor and other estuaries probably afforded even more cover and protection from rapid currents. Holding periods were apparently unrelated to tide stage or salinity, indicating that salinity did not present a barrier to smolt migration at Cow Point. However, pollutants entering the estuary in this region may have interfered with smolt migration (Seiler 1989).

Holding behavior served to slow seaward migration of coho salmon smolts. Smith (1982) found that migration rates for coho salmon smolts predicted from laboratory swimming performance experiments were much faster than actual rates determined from tag recaptures in the Columbia River, Washington. He speculated that this discrepancy could have resulted if smolts had only been actively swimming one third of the time. Our observations indicated that such behavior is likely, perhaps related to the low physiological capacity for continuous swimming observed in smolts (Glova and McInerney 1977; Flagg et al. 1983; Virtanen and Forsman 1987). In contrast with observations of nocturnal migratory behavior in Atlantic salmon smolts (Thorpe et al. 1981), data from our riverine monitoring stations suggested that coho smolts were most active during the day.

Time of release and water temperature apparently affected downstream movement of smolts within the river. However, smolt size did not seem to affect the migration behavior we observed, perhaps because we selected large smolts to avoid tag effects. Smolts released early in the season showed reduced downstream progress or actually swam upstream, indicating that they may not have been ready to begin seaward migration. Fried et al. (1978) described a similar "premigratory phase" for Atlantic salmon smolts. Since river current velocity decreased over our study period, greater downstream progress by late releases could not be attributed to enhanced passive transport. Delayed expression of migratory activity was probably related to the physiological condition of the smolts (Thorpe and Morgan 1978). Water temperature probably contributed to migration readiness (Wedemeyer et al. 1980) as evidenced by the elevated migration rates we observed during our warmest tracking days. Our data indicated that smolts released too early may not migrate normally, perhaps contributing to the high mortality that Mathews and Ishida (1989) observed for early hatchery releases of coho salmon smolts.

We found that swimming behavior of coho salmon smolts was most affected by current velocity. Ground speeds, estimated swimming speeds, and orientation to the current were all highly correlated with current velocity but not with current direction. These data indicated that coho salmon smolts did not use selective tidal stream transport, as has been described for some marine fishes (Arnold 1981). Similarly, LaBar et al. (1978) found no evidence for selective tidal stream transport in Atlantic salmon smolts. However, LaBar et al. (1978) did find that Atlantic salmon smolts seemed to switch from passively drifting with tidal currents to more active seaward migration upon entering the lower estuary. We did not observe such a change in migratory behavior of coho salmon smolts within the estuary.

Coho salmon smolts exhibited strong, positive rheotaxis when they encountered swift currents, but moved both with and against slow currents. This behavior resembled that of homing adult American shad (*Alosa sapidissima*) in estuaries (Dodson and Leggett 1973). During ebb tides in 1988 (the most rapid current conditions we observed), mean estimated smolt swimming speeds (2.5 body lengths·s^{-1}) exceeded theoretical optimum foraging (1.8 body lengths·s^{-1}) and cruising (1.4 body lengths·s^{-1}) speeds for fish of approximately the same size in 15°C seawater (Ware 1978). In more moderate current conditions, the coho smolt swimming speeds were much closer to theoretical optima. Consequently, our mean estimate of total energy (T) consumed by the migrating smolts (0.027 cal·s^{-1}) was almost twice Ware's (1978) T calculated for 20–cm fish feeding at their optimal swimming speed, 0.014 cal·s^{-1}. Finally, we found that the daily caloric expenditures for smolts in 1988 were nearly twice those of smolts which experienced lower current velocities in 1989. These data suggest that high flow conditions may increase the energetic demands of migrating smolts, in addition to altering their rates of downstream progress.

The coho salmon smolts in our study increased their estuarine residence by swimming most vigorously against outgoing currents and holding for extended periods. Prolonged estuarine residence has been documented for some other juvenile Pacific salmon species (*Oncorhynchus keta*, Mason 1974; *O. tshawytscha*, Healey 1980). Perhaps smolts require a staging period in estuaries to assemble information necessary for their return migration as adults, to adjust their osmoregulatory machinery (Iwata and Komatsu 1984; Zaugg et al. 1985), to take advantage of increased food availability (Healey 1980), or to postpone increasing their predation risk (Bax 1983). However, it should be noted that estuarine use varies among salmon species and races (Dawley et al. 1986).

Acknowledgments

We thank Steve Schroder, Kurt Fresh, and Mark Carr of the Washington Department of Fisheries for their support throughout this project and Dave Seiler and his staff (WDF) for their assistance in fish procurement. Special thanks are also due to Conrad Mahnken, Chuck Bartlett, and the National Marine Fisheries Service, Coastal Zone and Estuarine Studies Division for donating the radio transmitters, portable receivers, and remote monitoring units, to the Environmental Protection Agency for providing the boat used in river tracking, and to the U.S. Fish and Wildlife Service for providing the boat used in estuarine tracking. We also acknowledge Steve W. Ross for his help both in the field and with manuscript preparation and Selena Keehnal and others for help with fish tracking.

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Offshore Distribution, Size, Age, and Lateral Plate Variation of Late Larval/Early Juvenile Sticklebacks (*Gasterosteus*) off the Atlantic Coast of New Jersey and New York[1]

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Cowen, R. K., L. A. Chiarella, C. J. Gomez, and M. A. Bell. 1991. Offshore distribution, size, age, and lateral plate variation of late larval/early juvenile sticklebacks (*Gasterosteus*) off the Atlantic coast of New Jersey and New York. Can. J. Fish. Aquat. Sci. 48: 1679–1684.

Late larval and early juvenile sticklebacks, *Gasterosteus aculeatus* (14–28 mm SL) and *G. wheatlandi* (11–19 mm SL), were collected over shelf waters in the New York Bight during May, June, and July 1988. *Gasterosteus aculeatus* occurred up to 110 km offshore, with peak abundances found from 3 to 75 km. *Gasterosteus wheatlandi* was most prevalent within 10 km of shore, but some individuals were collected up to 55 km offshore. The further offshore distribution of *G. aculeatus* should increase its potential for dispersal, perhaps explaining their capacity to found freshwater populations in recently deglaciated habitats. The relatively nearshore distribution of *G. wheatlandi* should minimize dispersal (especially around Cape Cod) and may maintain a distinct, morphological step cline by the relative isolation of two subpopulations above and below Cape Cod.

Des individus en fin de phase larvaire et des juvéniles d'épinoches, *Gasterosteus aculeatus* (14–28 mm LS) et *G. wheatlandi* (11–19 mm LS), ont été recueillis en mai, juin et juillet 1988 au-dessus des eaux de la plate-forme continentale dans la baie de New York. *Gasterosteus aculeatus* était présente jusqu'à 110 km au large, avec des pics d'abondance entre 3 et 75 km. *Gasterosteus wheatlandi* se trouvait surtout à moins de 10 km du rivage, mais certains individus ont été capturés jusqu'à 55 km au large. La distribution plus océanique de *G. aculeatus* devrait augmenter son potentiel de dispersion, ce qui explique peut-être la capacité de l'espèce à fonder des populations dulcicoles dans des habitats dont la déglaciation est récente. La distribution relativement côtière de *G. wheatlandi* devrait réduire la dispersion (particulièrement autour du cap Cod) et peut maintenir une cline morphologique discontinue par l'isolement relatif de deux sous-populations au-dessus et au-dessous du cap Cod.

Received October 30, 1990
Accepted March 15, 1991
(JA779)

Reçu le 30 octobre 1990
Accepté le 15 mars 1991

The nominal species, *Gasterosteus aculeatus* (threespine stickleback), is a complex of morphologically diverse allopatric populations and biological species of coastal and lowland freshwaters in the Northern Hemisphere (Wootton 1976; Bell 1984). The biology of *G. aculeatus* is exceptionally well known, especially its behavior, evolutionary biology, ecology, and physiology, placing it among the most thoroughly studied of nonfood fish species. Almost all research on *G. aculeatus* has focused on freshwater populations, and the few studies of marine and anadromous populations have investigated their biology and reproduction while in tidal pools or freshwater environments. Although *G. aculeatus* has been observed at sea, these observations tend to be anecdotal, only based on a few individuals (Brown and Cheng 1946; Jones and John 1978; Taylor and McPhail 1986). Recently, however, Quinn and Light (1989) found large numbers of adult *G. aculeatus* in the open North Pacific.

In comparison, *G. wheatlandi* (blackspotted stickleback), a close relative of the *G. aculeatus* complex, is poorly known (Wootton 1976). *Gasterosteus wheatlandi* is endemic to the Atlantic coast of northern North America, rarely entering freshwater (Wootton 1976). It has not been reported from the open Atlantic Ocean, although this, in part, may be because it is easily confused with *G. aculeatus*. Two recent studies have reported on both species in the marine environment, although these studies were conducted in semiprotected waters. Picard et al. (1990) studied the distribution of both species in the St. Lawrence estuary and adjacent tidal streams and pools; Williams and Delbeck (1989) compared the distribution, diet, and growth of *G. aculeatus* and *G. wheatlandi* in the Bay of Fundy. The above studies suggest that, while only spawning within freshwater and/or littoral environments (Wootton 1976), sticklebacks may be more prevalent in marine waters than previously thought.

Knowledge of the movements of sticklebacks in marine waters is important to interpret their overall distribution. For example, *G. aculeatus* is primitively marine, and freshwater populations have been derived repeatedly from marine ancestors (Bell 1984, 1987, 1988). Understanding the extent of their occurrence within marine waters may help interpret their widespread distribution in freshwater. Similarly, knowledge of the

[1]Contribution No. 801 of the Marine Sciences Research Center and Contribution No. 782 of the Department of Ecology and Evolution.

offshore occurrence and distribution of *G. wheatlandi* may lend insight toward an explanation of this species' relatively restricted distribution. Additionally, Sargent et al. (1984) described the existence of a sharp morphological step cline in *G. wheatlandi*, with an apparent break occurring in the vicinity of Cape Cod. Studies of the sticklebacks' offshore distribution may help explain the cause and/or maintenance of this step cline.

In this paper, we examine the offshore distribution of both *G. aculeatus* and *G. wheatlandi* with respect to the distance they occurred offshore, the timing of their offshore movement, and the age of first appearance. We also report on the growth rates of the young stages found offshore, as well as various morphological measurements and observations of each species. These results are interpreted with respect to the physical oceanography of this region and the potential for colonization of freshwater by *G. aculeatus*.

Methods

Eight ichthyoplankton cruises were conducted in the New York Bight (Cape May, New Jersey, to Montauk, New York) from May through August 1988. A total of 327 stations were sampled along various transects running perpendicular to shore from 3 to 200 km offshore. All fish in this study were sampled using a 5-m^2 Methot Frame net with 2-mm mesh (Methot 1986). The net was towed at the surface (thus sampling the upper 2 m) for 10 min at a speed of 1.0–1.5 $m \cdot s^{-1}$. Fish were preserved in 95% ethanol. Measurements of temperature and salinity were recorded at each station with an internal recording STD (Applied Microsystems Inc, model AMS-STD 12).

Specimens were placed in a weak alkaline solution (KOH) of Alizarin Red S for 2–4 h to stain bony structures red (e.g. Bell 1984; Sargent et al. 1984). *Gasterosteus aculeatus* and *G. wheatlandi* were distinguished by presence in the latter of a large cusp at the base of the pelvic spine (Hubbs 1929). Bony lateral plates were counted to place each specimen into complete, partial, and low plate morphs (sensu Hagen and Gilbertson 1972, as modified by Sargent et al. 1984 for *G. wheatlandi*). The sum of plate counts on both sides of the body is reported. Standard length (SL) was measured using dial calipers and recorded to the nearest 0.1 mm. Otoliths from both species were removed from selected fish based on SL to ensure that all size classes were represented. Otoliths were mounted on glass slides using commercial fingernail hardener. Microincrements (putative daily rings, see below) were counted by viewing a digitized light microscopy video image (Optical Pattern Recognition System, OPRS, Biosonics, Inc.) at 400×. The otoliths were generally read whole, although occasionally, fine grinding was done on wet 1200 grit wet/dry sandpaper. Growth rates were determined from the slope of the linear regression of otolith increment number versus SL.

Results

A total of 549 sticklebacks were collected during five of the eight offshore cruises (Fig. 1). Nine *G. aculeatus* were caught offshore during the second cruise (21–23 May) and 28 were caught during the third cruise (1–3 June). Their abundance rapidly peaked (n = 392) in the samples from the fourth cruise (June 11–16). *Gasterosteus wheatlandi* were caught during only the fourth cruise on 11–16 June (n = 157). By 6 July (cruise 5), only two *G. aculeatus* were caught, and one was caught on 19 July (cruise 6). Surface water temperatures during this period ranged from 12 to 18°C and salinities ranged from 29 to 34 ppt.

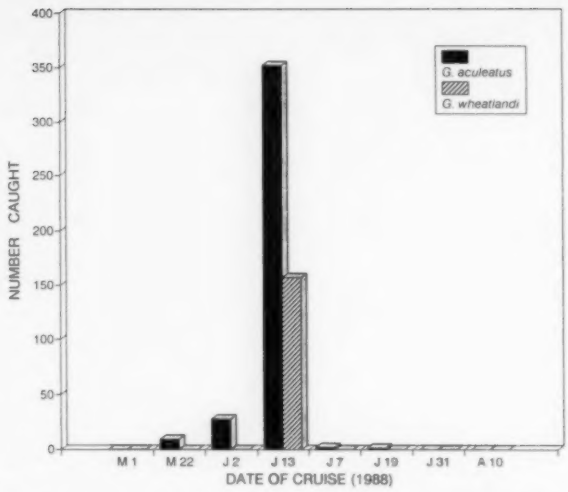


FIG. 1. Total number of *G. aculeatus* and *G. wheatlandi* caught during each cruise in 1988. Cruise dates: M 1, 24 April to 1 May; M 22, 21–23 May; J 2 1–3 June; J 13, 11–16 June; J 7, 6–9 July; J 19, 16–22 July; J 31, 29 July to 2 August; A 10, 8–12 August.

The offshore distributions of the two species were distinct. The more abundant *G. aculeatus* was caught up to 110 km offshore but were most abundant 3–75 km from shore (Fig. 2). It was distributed from our southern most sampling area off New Jersey to the eastern tip of Long Island. In contrast, *G. wheatlandi* was caught primarily within 33 km of shore, although two individuals were caught as far as 55 km offshore. *Gasterosteus wheatlandi* was collected primarily in the vicinity of central Long Island and was absent southwest of Long Island (Fig. 2).

Gasterosteus aculeatus was generally larger, older, and more variable in size than *G. wheatlandi*. The former ranged in size from 14 to 28 mm SL, with a mean size of 20 mm, whereas the latter ranged from 11 to 19 mm SL, with a mean size of 15 mm (Fig. 3). The otolith increments were assumed to be daily based on the finding that the back-calculated age at capture corresponded to expected birth dates for this area (Perlmutter 1963; Picard et al. 1990). Based on the increment counts, both species were very young when collected offshore. *Gasterosteus aculeatus* was between 29 and 56 d old, corresponding to birth dates in mid-April to mid-May. *Gasterosteus wheatlandi* ranged from 22 to 40 d old which corresponded to birth dates between early and mid-May. For the size ranges sampled, growth rates were not significantly different between species. The pooled growth rate was 0.47 $mm \cdot d^{-1}$ (Fig. 4).

Both species had a distinct coloration that differed from that of the typical golden, estuarine forms. The lateral flanks were silvery and the dorsal portion of their body was a dark blue, providing an obvious countershading effect. In addition, *G. aculeatus* had a caudal keel, which is absent in *G. wheatlandi*.

All *G. aculeatus* were complete plate morphs, with a continuous row of plates extending from the nape onto the caudal peduncle. In comparison, *G. wheatlandi* had a range of 20–60 lateral plates, with a mode of 56 (Fig. 5). Such counts represent primarily the partial–complete plate phenotype (Hagen and Gil-

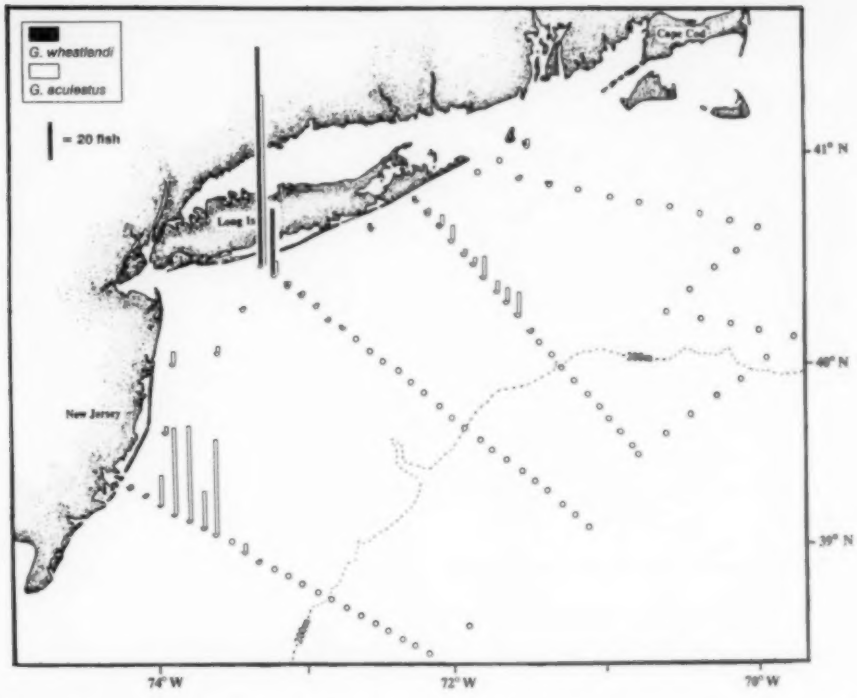


FIG. 2. Distribution of *G. aculeatus* (open bars) and *G. wheatlandi* (solid bars) within the New York Bight during cruise 4 (11–16 June 1988). All station locations are marked along each sampling transect. The number caught at each station is indicated by the height of the bar.

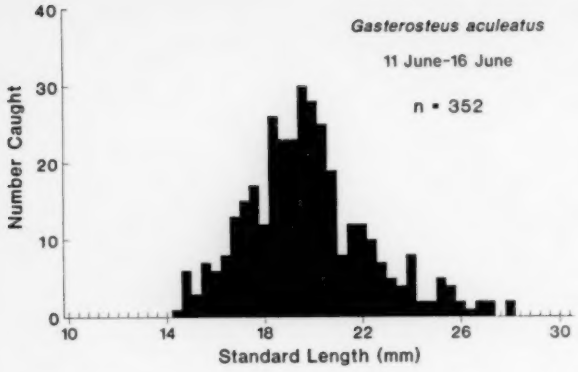
bertson 1972; Sargent et al. 1984). The proportion of low plate phenotypes in this study was 0.038. To determine if plate formation was complete at this young age, plate counts were plotted against SL (Fig. 6). There was no correlation between plate count and size, indicating that plate development is completed at a very young age. Because all specimens were very young juveniles, no sexual dimorphism in lateral plate counts could be determined.

Discussion

This study is the first documentation of an apparent offshore movement of late larval/early juvenile sticklebacks. In most studies reporting offshore occurrences of sticklebacks, the fish have been adults (see Jones and John 1978). Quinn and Light (1989) caught large numbers of sticklebacks in purse seines in the North Pacific Ocean up to 945 km from shore. Their findings demonstrated that offshore occurrence is not unusual, at least for adult fish. In the few recent studies where young-of-the-year (YOY) sticklebacks have been considered (Williams and Delbeck 1989; Picard et al. 1990), the movements were typically within a few kilometres of shore, still within the estuarine environment, and the fish were nearly twice the size (2–4 cm SL) of those collected in this study (*G. aculeatus*, 1.4–2.8 cm SL; *G. wheatlandi*, 1.1–2.0 cm SL).

Two points are of interest with respect to the offshore movement of young sticklebacks reported in this study. First is the large distance covered by these small fish and the relatively short time over which their movement occured. The large movement of sticklebacks offshore (from estuarine spawning habitat) primarily occurred between our third (1–3 June) and fourth (11–16 June) cruise, or approximately a 10- to 12-d period. Following an exercise similar to that of Quinn and Light (1989), we can calculate possible sustainable swimming capacities of sticklebacks using a conservative estimate of 3 body lengths (BL)·s^{-1} (e.g. Taylor and McPhail 1986; Whoriskey and Wootton 1987). At this speed, a 2-cm stickleback could swim approximately 50 km within 10 d. The slightly larger *G. aculeatus* could conceivably swim farther offshore than *G. wheatlandi*, which may explain the observed difference in their offshore distribution. The slight difference in morphology of *G. aculeatus*, including the presence of a caudal keel, also suggests that this stickleback may be a stronger swimmer than *G. wheatlandi*. Offshore flow patterns cannot explain the observed distribution, since the prevailing currents within the New York Bight are along the shelf rather than in an offshore direction (Bumpus 1973), and no significant wind events were recorded during the period involved (wind records from John F. Kennedy Airport, Long Island, NY).

As sticklebacks are primitively marine (Bell 1984, 1987, 1988), their offshore occurrence as adults should not be a surprise. However, they are believed to breed only in littoral and freshwater environments (Baggerman 1957; Wootton 1976; Audet et al. 1986); thus, their movement offshore at such a young age as found in the present study is perplexing. Perhaps predation pressure inshore is large on this size class of fish. For example, the timing of the offshore movement of these sticklebacks coincides with the inshore movement of the spring-spawned bluefish, *Pomatomus saltatrix* (Nyman and Conover 1988; R. K. Cowen, unpubl. data), a voracious piscivore.



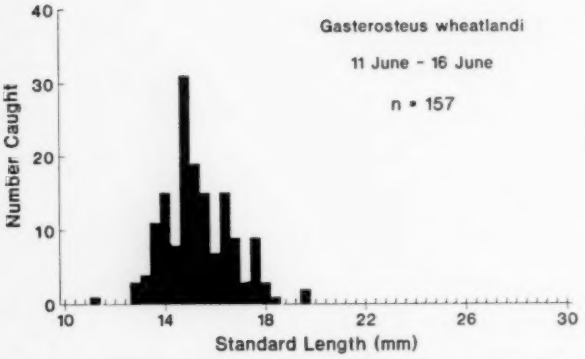


FIG. 3. Size frequency (SL) of all sticklebacks collected during cruise 4 (11–16 June 1988).

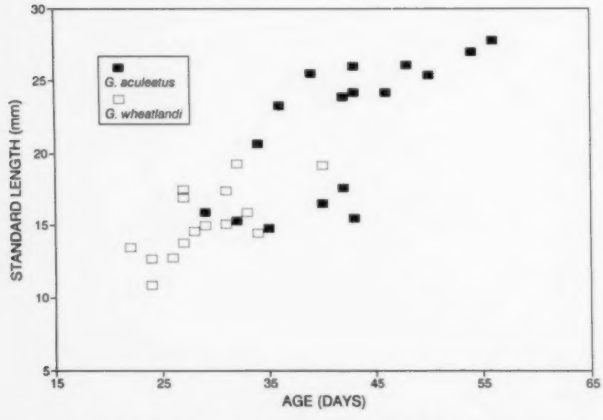


FIG. 4. Age to SL relationship for *G. aculeatus* and *G. wheatlandi*. Regression: SL = 1.85 + 0.47 (age), $r^2 = 0.72$, $p < 0.05$.

Additionally, Foster et al. (1988) found cannibalism to be a significant factor in causing an ontogenetic shift in habitat use by freshwater populations of *G. aculeatus*. Other possible

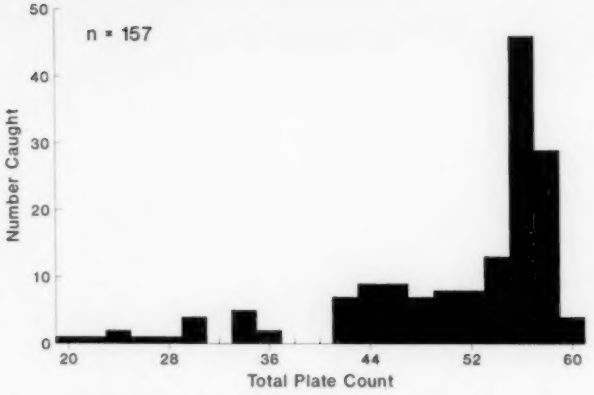


FIG. 5. Distribution of total plate counts of *G. wheatlandi* collected during cruise 4 (11–16 June 1988).

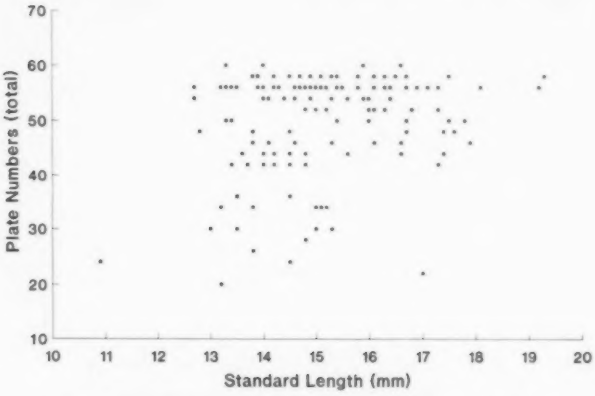


FIG. 6. Total plate counts of *G. wheatlandi* by size (SL).

advantages of moving offshore include increased growth rates, but there is yet insufficient information to compare our observed growth rate estimates with those of sticklebacks in other habitats.

It is unclear where the sticklebacks go after their brief, but abundant appearance offshore in mid-June. *Gasterosteus* spp. apparently are absent from inshore and freshwater habitats outside the summer breeding season. Conceivably, they remain offshore, but are no longer near the surface. Several workers have reported the collection of stickleback adults in offshore waters at depths up to 200 m (Jones and John 1978). J. D. McPhail (Department of Zoology, University of British Columbia, pers. comm.) reported that large numbers of adult sticklebacks are associated with the deep scattering layer in waters off Vancouver Island, Canada. Another possibility is that the fish return to the inshore estuaries. If so, this might indicate that we happened to witness an unusual one-time event, possibly climatically induced, that caused the young sticklebacks to move offshore. However, sampling in 1989, although not as extensive as in 1988, also found young sticklebacks up to 100 km from shore.

The second point of interest is the implication of this offshore distribution on the population structure of these fish. The nearshore distribution of *G. wheatlandi* may provide some insight as to why there is a distinct break in lateral plate phenotypes

that Sargent et al. (1984) reported at Cape Cod. Within the Gulf of Maine, water flows along shore in a southward direction and then veers eastward as it impinges on the Cape Cod peninsula (Bumpus 1973; Beardsley et al. 1976). While some of this water may turn south and flow around the Cape into the New York Bight (Sutcliffe et al. 1976), the prevailing flow is eastward and then northward in a counterclockwise recirculation of the Gulf. The shelf waters south of Cape Cod flow southwest towards Long Island (Bumpus 1973; Beardsley et al. 1976). Given this circulation pattern, and the relatively nearshore distribution of *G. wheatlandi*, we can speculate that these fish are not readily transported around Cape Cod from the Gulf of Maine into the New York Bight. Therefore, a distinct step cline is maintained by the relative isolation of the two sub-populations above and below Cape Cod.

Why *G. wheatlandi* does not occur further west or southwest than Long Island remains a perplexing question, especially given its offshore occurrence and the prevailing southwest surface current. Perhaps the time *G. wheatlandi* spends in the surface waters is so limited that advection to the southwest is minimal. Instead, the fish may enter the deeper waters below the thermocline, which do not move in a southwesterly direction (Bumpus 1973). Other explanations may include both ecological and/or physiological requirements that are not met in habitats further to the south. This question remains open to further study.

In comparison with *G. wheatlandi*, *G. aculeatus* is widely distributed throughout coastal freshwater habitats in subarctic to temperate regions of the Northern Hemisphere (Wootton 1976; Bell 1984, 1987). This species' present distribution includes freshwater habitats that were glacially defaunated (i.e. islands, isolated headlands, fiords). Since these habitats could not be recolonized through freshwater, they required repeated colonization by marine populations (Bell 1984, 1987). Although there is circumstantial evidence that anadromous *G. aculeatus* homes to its spawning grounds (Hagen 1967), our observation of large numbers of specimens at least 110 km offshore indicates that it regularly moves long distances from its inshore or freshwater breeding sites during its life cycle. This movement far offshore could easily provide the opportunity to stray into recently deglaciated habitats and establish freshwater populations.

Finally, lateral plate formation occurred very early in the populations we studied as compared with those in other areas. Lateral plates appear first above the pelvic girdle and then on the caudal peduncle during larval ontogeny of *G. aculeatus* complete plate morphs. Plates are added in sequence to the proximal ends of the two plate rows until the area between them is filled. Plate development begins at about 13 mm total length and all plates are present by 24–27 mm in an anadromous complete morph population from Japan (Igarashi 1970). In contrast, onset and termination of plate addition do not occur until 15 and 30–36 mm total length, respectively, in freshwater complete plate morphs from Japan (Igarashi 1964). Plate development also terminated at about 30 mm SL in freshwater complete plate morphs from California (Bell 1981). At 14 mm SL, our smallest *G. aculeatus* had completed plate development. Clearly, the size at which plate development occurs varies among populations. These studies suggest that plate development occurs earlier in marine and anadromous populations than in freshwater *G. aculeatus*, but more populations must be sampled before associations between habitat and the rate of plate development can be established.

Plate development proceeds faster in *G. wheatlandi* than in *G. aculeatus* in our samples. Thus, reduction of plate number and presence of gaps in the plate row of *G. wheatlandi* (Sargent et al. 1984) must be due to earlier termination of plate development. Assuming that the complete plate morph represents the primitive condition for *Gasterosteus*, reduced numbers of plates in *G. wheatlandi* represent paedomorphosis by means of early termination of plate development, or progenesis (Alberch et al. 1979). Low and partial plate morphs of *G. aculeatus* also appear to be paedomorphs of the complete plate morph, but in populations studied by Bell (1981) the main (i.e. 92.4%) cause of lateral plate paedomorphosis was a lower rate of plate addition, or neoteny (Alberch et al. 1979). If this difference in the developmental basis for plate reduction is species specific, it seems likely that low and partial lateral plate phenotypes have evolved independently in the two species. Thus, although the propensity to evolve low and partial plate phenotypes may be a synapomorphy of *Gasterosteus*, evolution of reduced plate phenotypes represents homoplasy. Convergent evolution of lateral plate phenotypes in these species is not unexpected because iterative homoplasy of lateral plate (Bell 1981, 1984) and pelvic girdle phenotypes (Bell 1987) is common within the *G. aculeatus* species complex.

Acknowledgements

Mike Fahay (NMFS, Sandy Hook, NJ) originally alerted us to the uniqueness of finding young sticklebacks offshore and we thank him for discussions on this topic. We thank both Su Sponaugle and Jon Hare (MSRC, SUNY, Stony Brook, NY) for critical reviews of the manuscript. This work is a result of research sponsored by the NOAA Office of Sea Grant, U.S. Department of Commerce, under grant No. NA86AA-D-SG045 to the New York Sea Grant Institute and from the NYDEC through the Dingell-Johnson Federal Aid in Sport Fish Restoration Act.

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Bioassay-Derived 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin Equivalents in PCB-Containing Extracts from the Flesh and Eggs of Lake Michigan Chinook Salmon (*Oncorhynchus tshawytscha*) and Possible Implications for Reproduction

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Ankley, G. T., D. E. Tillitt, J. P. Giesy, P. D. Jones, and D. A. Verbrugge. 1991. Bioassay-derived 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalents in PCB-containing extracts from the flesh and eggs of Lake Michigan chinook salmon (*Oncorhynchus tshawytscha*) and possible implications for reproduction. Can. J. Fish. Aquat. Sci. 48: 1685–1690.

Concentrations of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalents (TCDD-EQ), derived via the H4IIE rat hepatoma cell bioassay, were measured in polychlorinated biphenyl (PCB) containing extracts of flesh (dorsal muscle) and egg samples from 10 spawning chinook salmon (*Oncorhynchus tshawytscha*) from Lake Michigan. There was a marked maternal transfer of both TCDD-EQ and PCBs, and potency of the PCB mixture (expressed as picograms of TCDD-EQ per microgram of PCB) in eggs was 2.5 times greater than potency of the PCB mixture in dorsal muscle of the fish. There was a statistically significant, inverse relationship between the total concentration of PCBs in eggs and hatching success of the fish, with an effect concentration that corresponded to approximately 100 pg TCDD-EQ/g egg. Our results, based on a relatively small sample size, suggest that PCBs, in particular those with TCDD-type activity, may have influenced reproductive success of the fish.

On a mesuré les concentrations des équivalents de 2,3,7,8-tétrachlorodibenzo-*p*-dioxine (TCDD-EQ) obtenus par bioessai cellulaire H4IIE sur les hépatomes de rat, dans des extraits de chair (muscle dorsal) et des échantillons d'oeufs de dix géniteurs de saumon quinnat (*Oncorhynchus tshawytscha*) du lac Michigan contenant des BPC biphényles polychlorés (BPC). On a observé un transfert maternel marqué à la fois des TCDD-EQ et des BPC, et l'activité du mélange de BPC (exprimé en picogrammes de TCDD-EQ par microgramme de BPC) dans les oeufs était 2,5 fois supérieure à l'activité de ce mélange dans le muscle dorsal du poisson. Il existait une relation inverse, statistiquement significative, entre la concentration totale de BPC dans les oeufs et le succès d'éclosion des poissons, avec une concentration efficace correspondant à environ 100 pg TCDD-EQ/g oeufs. Nos résultats, fondés sur un échantillon relativement petit, peuvent indiquer que les BPC, en particulier ceux dont l'activité est de type TCDD, peuvent agir sur le succès de reproduction des poissons.

Received November 6, 1990
Accepted March 15, 1991
(JA792)

Reçu le 6 novembre 1990
Accepté le 15 mars 1991

Although polychlorinated biphenyls (PCBs) have been monitored in the environment for a number of years, only recently has it been possible to reliably identify and quantify the planar congeners in the PCB mixture. Recent studies have demonstrated the presence of the planar PCBs 3,3′,4,4′-tetrachlorobiphenyl, 3,3′,4,4′,5-pentachlorobiphenyl, and 3,3′,4,4′,5,5′-hexachlorobiphenyl (IUPAC Nos. 77, 126, and 169) in sediments, invertebrates, fishes, waterfowl, and aquatic mammals from a variety of freshwater and marine sites (Huckins et al. 1988; Kannan et al. 1989a, 1989b, 1989c; Kubiak et al. 1989; Niimi and and Oliver 1989a). Based on structure activity and toxicity data from mammalian studies, it has been proposed that these and other planar congeners potentially contribute the majority of the toxic potency to PCB mixtures and thus are of great toxicological significance in aquatic ecosystems (Tanabe et al. 1987; Kannan et al. 1989b; Kubiak et al. 1989).

Although it is possible to determine concentrations of planar PCBs, there still are many uncertainties as how best to assess the toxicity of mixtures that can contain more than 100 planar and nonplanar congeners which may interact with one another. Some researchers have used toxic equivalency factors (TEF), based upon potency of different congeners relative to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), in an additive index, to express the total toxic potential of mixtures of PCBs and related compounds such as polychlorinated dibenzofurans (PCDFs) and polychlorinated dibenzodioxins (PCDDs) (Sawyer and Safe 1985; Eadon et al. 1986; Kannan et al. 1988; Kannan et al. 1989b; Niimi and Oliver 1989b; van Zorge et al. 1989; Bellward et al. 1990; Olafson et al. 1990). This approach is reasonable because planar PCBs and the toxic 2,3,7,8-substituted PCDFs and PCDDs (for which TCDD is the prototype compound) all appear to have the same mode of action (Neal 1985);

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however, there are important uncertainties associated with the approach. For example, because there is a limited database on the relative potency of some congeners, in many instances it is difficult to assign realistic potency values to individual PCBs. Moreover, there is no assurance that an additive model is the best predictor of toxicity of mixtures of planar and nonplanar PCBs. Dependent upon exposure conditions, studies with different combinations of PCBs, PCDFs, and PCDDs suggest that interactions among congeners may range from antagonism (Bannister et al. 1987; Biegel et al. 1989) to additivity (Sawyer and Safe 1985; Weber et al. 1985; Birnbaum et al. 1987; Pluess et al. 1988), to, perhaps, synergism (Birnbaum et al. 1985; Bannister and Safe 1987). Ideally, from a hazard assessment standpoint, a simple measure of the biological potency of complex mixtures of PCBs and/or complex mixtures of PCBs, PCDFs, and PCDDs would be extremely useful in obviating problems associated with the assumptions involved in "traditional" TCDD-TEF evaluations. Also, it might be predicted that an integrated biochemical measure of potency would be a reasonable proximate predictor of the biological effects of these types of compounds.

In the late 1970s, researchers with the U.S. Food and Drug Administration suggested that a simple bioassay, based upon measurement of the induction of cytochrome P-450-associated monooxygenase activity in the H4IIE rat hepatoma cell line, could be a useful tool in determining the presence of TCDD and similar compounds in environmental samples (Bradlaw and Casterline 1979; Bradlaw et al. 1980; Casterline et al. 1983). In subsequent studies, Safe and co-workers have demonstrated that induction of either arylhydrocarbon hydroxylase or 7-ethoxyresorufin-*O*-dethylase (EROD) activity in the H4IIE cell line by PCB, PCDF, and PCDD congeners, either singly or in combination, correlates well with the in vivo toxicity of these compounds to rats (Bandiera et al. 1984; Leece et al. 1985; Mason et al. 1985, 1986; Safe et al. 1987, 1989). More recently, Zacharewski et al. (1989) and Tillitt et al. (1989, 1991a, 1991b) demonstrated the utility of the H4IIE bioassay for assessing the potency of various combinations of complex mixtures of PCBs, PCDFs, and PCDDs in samples of fishes and birds from the Great Lakes.

The objective of this study was to use the H4IIE bioassay to determine the biological potency, relative to TCDD, of the PCB mixture in extracts of flesh and egg samples from female chinook salmon (*Oncorhynchus tshawytscha*) from Lake Michigan. This particular focus was chosen because previous studies had documented adverse reproductive impacts, which were hypothesized to be due to contaminants such as PCBs, in both chinook salmon and lake trout (*Salvelinus namaycush*) from Lake Michigan (Mac 1988). In addition to collecting information concerning the potency and concentrations of PCBs in flesh and egg samples from the fish, correlations between PCB potency or concentration and reproductive success were examined.

Materials and Methods

Sample Collection and Reproduction

A detailed description of collection of the fish and evaluation of their reproductive success is given elsewhere (Ankley et al. 1989a, 1989b). Briefly, 30 female chinook salmon were collected and their eggs and flesh sampled. Triplicate subsamples of fertilized eggs from each fish were incubated and hatching success and fry survival to swim-up were determined. For the present study, samples of flesh and eggs from a subset of 10 of the fish, five exhibiting the best and five exhibiting the worst reproductive success, were selected for further evaluation.

Chlorinated organic hydrocarbons in samples of dorsal muscle and eggs were extracted by the method of Ribick et al. (1981, 1982). This consisted of initially grinding the samples with anhydrous sodium sulfate, followed by column extraction with dichloromethane, gel permeation chromatography, Florisil and silica gel clean-up steps, and, finally, for samples used in the H4IIE bioassay, solvent transfer into isooctane. This technique is quite specific for PCBs; the final fraction is free of detectable PCDFs, PCDDs, and polar pesticides and contains only a few of the organochlorine pesticides (Ribick et al. 1981, 1982). Although it was impossible to characterize extraction efficiency for the PCBs (i.e. spiked compounds could interfere with results of the bioassay), efficiencies on the order of 90–98% for the method have been reported (Ribick et al. 1981, 1982), and in our experience, efficiencies for the extraction of PCBs from fish flesh and eggs are quite similar (data not shown).

Cell Culture and Bioassay Procedure

Detailed descriptions of cell culture and enzyme assay techniques were given by Tillitt et al. (1991b). Briefly, H4IIE cells were seeded into Petri dishes (15 × 100 mm) at 0.8×10^6 cells/plate in 10 mL of culture media. After a 24-h incubation period, plates were dosed with four concentrations of each tissue extract, five concentrations of TCDD (1, 10, 25, 100, and 250 pg TCDD/plate) as a standard curve, or isooctane as a solvent blank. The amount of isooctane in all treatments was 1%, a concentration that does not elicit overt toxicity or affect enzyme induction in the cells (Tillitt et al. 1991b). Dosed cells were incubated for 72 h and were then rinsed with phosphate-buffered saline and harvested into Tris–sucrose (0.05–0.2 M) buffer. Protein in the cell suspensions was determined using the method of Lowry et al. (1951), and the cells were diluted to a protein concentration of 1 mg/mL with the Tris-sucrose buffer. EROD activity was determined by the method of Pohl and Fouts (1980). Final reaction mixtures contained (in 0.1 M HEPES buffer, pH 7.8) 5 mM glucose-6-phosphate, 5 mM $MgSO_4$, 3.5 mM NADP, 2.5 units of glucose-6-phosphate dehydrogenase, 100 μg of cellular protein, and 0.05 mL of 15 μM 7-ethoxyresorufin (in methanol) in a total volume of 1.25 mL. Incubations were conducted in a shaking water bath for 10 min at 37°C and were terminated by the addition of 2.5 mL of methanol. Resorufin in the supernatant was determined fluorimetrically (550 nm excitation, 585 nm emission) and quantified by comparison with a resorufin standard.

PCB Determination

Total concentrations of PCBs in tissue and egg extracts were determined using gas chromatography with electron capture detection (GC-ECD) and quantified using the method of Burkhard and Weininger (1987). These extracts were splits of the final silica gel fraction used for the H4IIE bioassay and were spiked with 2,4,6-trichlorobiphenyl (IUPAC No. 30) as a quantification standard prior to GC-ECD.

Statistical Analysis

Concentrations of TCDD equivalents (TCDD-EQ) in the flesh and egg extracts were determined using the slope-ratio

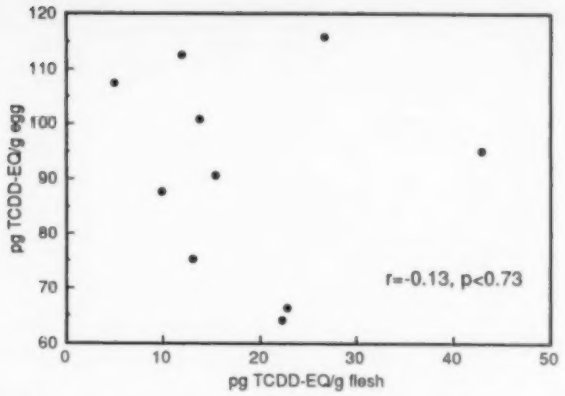

FIG. 1. Relationship of concentrations of TCDD-EQ in flesh to concentrations of TCDD-EQ in eggs from 10 Lake Michigan chinook salmon.

method of Finney (1978) by comparison with standard curves generated with TCDD. The slope-ratio method, rather than the approach described by Tillitt et al. (1991b), was used in the present study because maximal enzyme induction was not observed in cells dosed with the flesh extracts. The limit of quantification (LOQ) for the flesh and egg samples for this study was approximately 5 pg TCDD-EQ/g wet weight tissue.

Statistical manipulations, including Pearson pairwise correlations, simple linear regression, and *t*-tests, were performed using a standard computer package (Ryan et al. 1985).

Results and Discussion

TCDD-EQ were at detectable concentrations in all instances except in one flesh sample for which, although there appeared to be some enzyme induction, the slope of the dose–response curve was not significantly different from zero. In subsequent data summaries and statistical analyses, this sample was assigned a value of 2.5 pg TCDD-EQ/g (1/2 LOQ; Haas and Schett 1990).

The mean (± standard deviation) concentration of TCDD-EQ in the chinook salmon egg samples was 91.6 ± 18.4 pg/g, while the corresponding value in the flesh samples was approximately 5 times lower at 18.4 ± 11.2 pg/g. On a fish-by-fish basis, there was no apparent relationship between TCDD-EQ in the eggs and in the flesh (Fig. 1). Concentrations of TCDD-EQ in flesh of the chinook salmon were similar to those reported by Zacharewski et al. (1989), who used the H4IIE bioassay to determine the potency of extracts of chlorinated hydrocarbons from the flesh of fish from several of the Great Lakes, including Lake Michigan. Concentrations of TCDD-EQ in both egg and flesh samples from the chinook salmon were less (as much as an order of magnitude) than H4IIE-derived concentrations of TCDD-EQ in eggs of fish-eating birds such as terns and cormorants from Great Lakes sites known to be contaminated with PCBs, PCDFs, and PCDDs (Tillitt et al. 1989; 1991a).

Total PCBs were easily detectable by GC-ECD in all of the flesh and egg samples. The mean (± standard deviation) concentration of PCBs in the eggs was 3.80 ± 1.06 μg/g wet weight, while the mean concentration in samples of flesh was approximately 2.5 times lower at 1.50 ± 0.44 μg/g. Total concentrations of PCBs in both eggs and flesh were comparable with those recently reported for other collections of chinook salmon from the eastern shore of Lake Michigan (Giesy et al. 1986; Williams et al. 1989). As was true for concentrations of TCDD-EQ, there was no definable relationship, on a fish-by-fish basis, between concentrations of PCBs in eggs and flesh of the female salmon (data not shown).

There were strong, statistically significant linear relationships between concentrations of TCDD-EQ and PCBs in both the egg and dorsal muscle samples (Fig. 2). This provides evidence for the dominant contribution of PCBs to the concentrations of TCDD-EQ in these chinook salmon extracts.

The fact that TCDD-EQ measured in the eggs were 5 times greater than TCDD-EQ in the dorsal muscle, while total PCBs were only 2.5 times greater, is interesting. This suggests that if PCBs were the only compounds in the samples determining TCDD-EQ, the PCB mixture in the eggs was enriched relative to that in the flesh. To quantify this observation, the concentration of TCDD-EQ was divided by the concentration of PCBs in each sample. Thus, potency of the mixture is expressed as TCDD-EQ per unit PCBs. On this basis, the flesh samples had a mean (± standard deviation) potency of 11.80 ± 4.23 pg TCDD-EQ/μg PCB, while the egg samples were significantly greater ($t = 6.7$, $p < 0.0001$) at 24.75 ± 4.42 pg TCDD-EQ/μg PCB. Again, assuming that PCBs were the primary contributors to TCDD-EQ in the samples, this indicates that potency of the PCB mixture was about 2.5 times greater in eggs

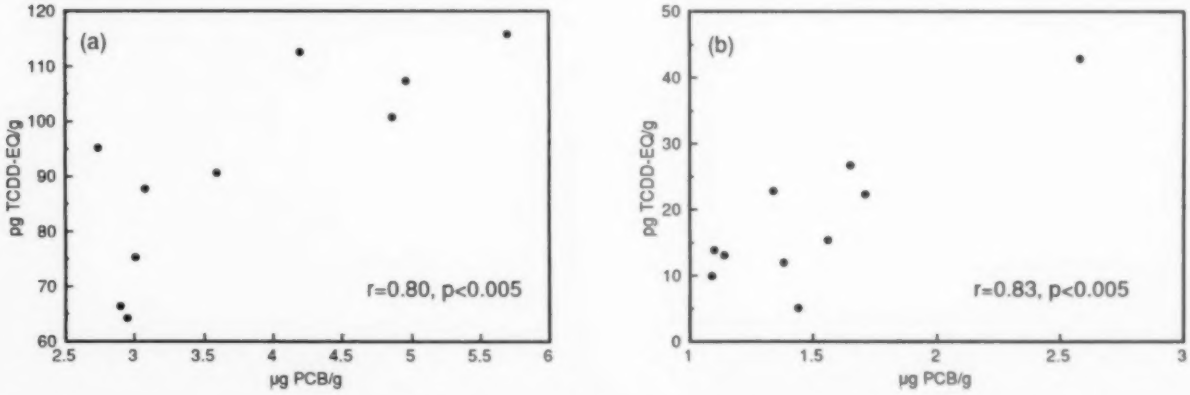

FIG. 2. Relationship between concentrations of TCDD-EQ versus PCBs in (a) eggs and (b) flesh from 10 Lake Michigan chinook salmon.

TABLE 1. Correlations of concentrations of TCDD-EQ and PCBs in eggs and flesh with hatching success and fry survival for chinook salmon from Lake Michigan.

Contaminant measure	Reproductive parameter	
	Hatch	Fry survival
TCDD-EQ (flesh)	0.46 (0.18)[a]	0.26 (0.50)
TCDD-EQ (eggs)	−0.51 (0.13)	−0.37 (0.33)
PCBs (flesh)	0.17 (0.64)	0.34 (0.37)
PCBs (eggs)	−0.75 (0.01)	−0.57 (0.11)

[a] r(prob > r).

as compared with dorsal muscle. Recently, others have found that enrichment of PCB potency (determined via the H4IIE bioassay; Tillitt et al. 1991a), as well as concentrations of planar PCB congeners (Kannan et al. 1989a), relative to original PCB mixtures (e.g. Aroclors), can occur in biota. However, this is the first observation that differences in PCB potency could occur among different tissues within an organism. An extremely important implication of any sort of enrichment of PCB potency is, of course, that hazard assessments based upon total PCBs will be inaccurate.

There are at least two possible explanations for the observation of enhanced PCB potency upon maternal transfer. It may be that concentrations of planar PCB congeners were greater in eggs as compared with flesh. Alternatively, patterns of planar and nonplanar PCBs in eggs versus flesh may have been altered so as to change toxicological interactions among the congeners. For example, some nonplanar PCB congeners may act as antagonists to planar PCBs (e.g. Biegel et al. 1989); thus, if the eggs contained smaller concentrations of antagonistic congener(s) than the flesh, even with similar concentrations of the planars, the egg mixture may nonetheless have appeared more potent. More extensive chemical analyses than conducted in this study would be required to further elucidate either of these or other possible explanations for the observed differences in apparent potency of PCBs in eggs versus flesh.

Among eight correlations of the concentrations of TCDD-EQ or PCBs in eggs or flesh with two measures of reproductive success in the fish (Table 1), the only significant one was an inverse relationship between hatching success and concentrations of PCBs in eggs (Fig. 3a). The relationship between percent hatch and concentrations of TCDD-EQ in the eggs (Fig. 3b) was not significant but was interesting in that there appeared to be a threshold, with hatching success consistently reduced at egg TCDD-EQ concentrations greater than 100 pg/g. This observation may be of particular significance in light of recent findings by researchers from the University of Wisconsin, who exposed lake trout eggs to TCDD in the water. In their studies, it was found that the LD50 concentration (in eggs) for fry mortality was approximately 65 pg TCDD/g (R. Peterson, pers. comm.), a value reasonably comparable with TCDD-EQ concentrations associated with adverse effects in the chinook salmon eggs from our study.

Due to uncertainties concerning the exact composition of the PCB mixture in the eggs of the chinook salmon, it is difficult to compare our data with the results of other studies evaluating relationships between PCBs and reproductive success in fishes (e.g. Hansen et al. 1973; Nebeker et al. 1974; Hogan and Brauhn 1975; Broyles and Noveck 1979; Willford et al. 1981; Mac 1988). It is worth noting, however, that unlike our observations, Stauffer (1979) found no relationship between total PCB concentration in eggs and reproductive success in chinook salmon from Lake Michigan, but again, the composition of the PCB mixture (as well as other contaminants) present in fish from the mid-1970s probably was quite different than the present array of chemicals in Lake Michigan chinook salmon.

Our results suggest that PCBs, in particular the TCDD-related activity of PCBs, may have been related to observed adverse reproductive impacts in these Lake Michigan chinook salmon; however, our sample size of 10 is far too small to justify using the present study as conclusive evidence of this possibility on a lake-wide basis. Moreover, it is impossible to eliminate possible effects of other contaminants likely present in the fish. Further studies are required to corroborate (or refute) these results. For example, it would be desirable to examine reproductive success versus PCB and TCDD-EQ concentrations in chinook salmon from other locations in Lake Michigan to develop a wider dose–response range. Also, further laboratory studies with planar PCBs, PCDFs, and PCDDs (including TCDD) should be performed to provide a database for assessing TCDD-EQ (or TCDD-TEF) concentrations in terms of toxicity to fishes. Finally, it is important to focus research efforts upon documenting and explaining the apparent phenomenon of selective enrichment of PCB potency between different environmental and biological compartments. The H4IIE bioassay should prove to be an excellent tool for these types of studies.

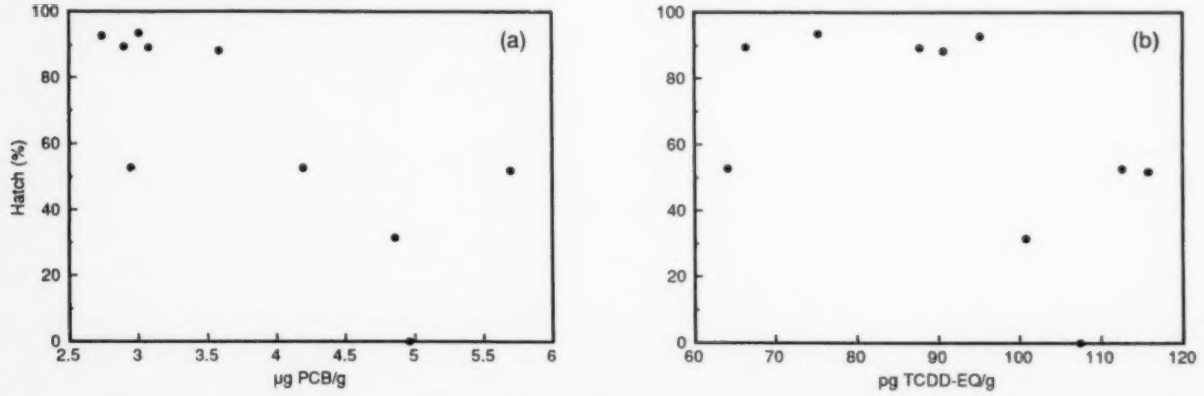

FIG. 3. Relationship of hatching success to concentration of (a) total PCBs and (b) TCDD-EQ in eggs from 10 Lake Michigan chinook salmon.

Acknowledgements

We thank all the persons who have contributed to this study. They include D. Ankley, R. Hoke, L. Williams, J. Newsted, C. Rosiu, and K. Obermeyer. We also thank the Michigan Department of Natural Resources for their cooperation in collection of the fish. Dr. P. Cook provided helpful comments on an earlier draft of the paper. D. Williams typed the manuscript and R. LePage prepared the figures. Support for portions of this work came from the Center for Environmental Toxicology at Michigan State University. This work was also supported with funds from grant No. NA 89AA-D-SG083 from the National Sea Grant College Program, National Oceanographic and Atmospheric Administration, U.S. Department of Commerce, and funds from the State of Michigan.

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Stock Abundance Estimation Using Depth-Dependent Trends and Spatially Correlated Variation

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Hydroacoustic data commonly contain a large number of observations that are correlated in space and time. Such data are complicated to analyze, and a good estimate of the error in abundance is often difficult to obtain. Hydroacoustic data collected on Shelikof Strait walleye pollock (*Theragra chalcogramma*) are used to develop statistical techniques for analyzing survey data containing spatial trends and spatial correlations. The data show a trend in fish density with depth, while detrended observations exhibit spatial correlation. Spatial means and variances of fish density, and total abundance and its variance, are determined using the geostatistical theory of kriging. The total abundance estimate is the same order of magnitude as estimates arrived at using a stratified estimation approach. Detrending the data with respect to depth through depth stratification decreased the estimated variance by nearly a factor of 10. Detrending the data with respect to depth and kriging the residual variation over space reduced the variance by an additional factor of 2. Modeling trends and making more efficient use of the data contribute to the gain in information

Les données hydroacoustiques renferment souvent un grand nombre d'observations corrélées dans l'espace et le temps. L'analyse de telles données est compliquée et il est souvent difficile d'obtenir une bonne estimation de l'erreur en matière d'abondance. L'auteur s'est servi de données hydroacoustiques sur la goberge de l'Alaska (*Theragra chalcogramma*) recueillies dans le détroit de Shelikof pour élaborer des méthodes d'analyse statistique de données de relevés renfermant des tendances et des corrélations spatiales. Les données révèlent une tendance de la densité des poissons liée à la profondeur, tandis que les observations dissociées montrent une corrélation spatiale. Les moyennes et les variances spatiales de la densité, ainsi que l'abondance totale et sa variance, sont déterminées à partir de la théorie géostatistique du krigeage. L'estimation de l'abondance totale est du même ordre de grandeur que les estimations obtenues selon la méthode d'estimation stratifiée. La dissociation des données par rapport à la profondeur par stratification des profondeurs a entraîné une baisse de la variance estimative par un facteur s'approchant de 10. D'autre part, la dissociation des données par rapport à la profondeur et le krigeage de la variance résiduelle en fonction du temps ont entraîné une baisse de la variance par un autre facteur de 2. La modélisation des tendances et l'utilisation plus efficace des données ajouteront aux connaissances acquises.

Received March 16, 1990
Accepted March 13, 1991
(JA508)

Reçu le 16 mars 1990
Accepté le 13 mars 1991

The assessment of fish population abundance from hydroacoustic survey data is often complicated by the spatial and temporal correlation among observations. Several approaches have been taken to deal with this problem. These include those based on classical statistics (e.g. stratified random sampling) and those based on time series analysis. For example, one random sampling approach places transects parallel to one another and at random distances apart over the survey area, treating each transect as a randomly sampled observation (Jolly and Hampton 1990). Under this approach, stratification according to expected fish density may be used to increase the precision of the estimates. Alternatively, other approaches make use of within-transect observations by taking into account the correlation between observations along a transect (e.g. see Nickerson and Dowd 1977). Under this approach, more of the available information is used but placement of the transects and the overall sampling design may not be specifically addressed, leading to possible estimation biases. In both approaches, questions may be raised with regard to appropriate survey designs, reduction of variance, and the cost effectiveness of conducting surveys in areas where there are little or no fish in order to preserve a specified sampling regime. Recently, alternatives that lie somewhere between these two statistical approaches have been explored (Guillard et al. 1990; Crittenden 1989).

In this paper, hydroacoustic data from surveys conducted on Shelikof Strait walleye pollock (*Theragra chalcogramma*) are detrended using depth as an auxiliary variable. The residual error is then modeled using the method of kriging, a method which has found wide application in the field of geostatistics (Journel and Huijbregts 1978). The overall objective of this study is to develop better statistical techniques for estimating fish population densities using data collected from hydroacoustic surveys. Certain characteristics of data collected by hydroacoustic sampling techniques, namely the large number of observations over the sample region and the degree of correlation between data points, often complicate the estimation of abundance when classical estimation techniques are employed. These same characteristics can be used to improve

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estimates of abundance, improve estimates of the variance in abundance, and improve sampling design when the existing spatial relationships in fish density are taken into account. Some specific objectives of this study follow: (1) examine data for evidence of spatial structure that can be used to reduce uncertainty in abundance estimation; (2) develop models, analytical techniques, and sampling schemes that take into account this spatial structure in order to get better estimates.

An analysis is conducted on data collected during the 1987 Shelikof Strait hydroacoustic survey. The data are examined for correlations between the observed densities and between changes in density and changes in the observed auxiliary information available on depth, date, time of day, and spatial location. An empirical relationship and a model are developed to describe the observed structure in terms of trend and spatial correlation. The technique of kriging is applied to the data, detrended using a density-depth relationship, to assist in determining spatial estimates of abundance and the associated spatial variances. Finally, it is determined if variability in the abundance estimates is affected by the observed structure in the data (e.g. trend and correlation). This work is seen as one step in the development of more sophisticated methods for the analysis of hydroacoustic survey data.

Data Analysis

Hydroacoustic surveys were conducted in Shelikof Strait, Alaska, in March of 1987 (Nunnallee 1988). The data were collected with a 38-kHz echo sounder and a digital echo integrator. The transducer was towed at an average depth of 21 m 24 h per day along a predetermined set of zigzag transect lines. The average vessel speed was 9 knots and the echo integrator outputs of fish density were averaged over 2-min running time segments for each of up to 35 contiguous 10-m depth intervals from 25 m below the transducer to within 3 m of the seabed.

Figure 1 shows the zigzag pattern from the March 25–29 sampling survey. Each point on the transect line represents one 2-min segment, and the height of the vertical line at that point represents the relative number of fish summed over depth occurring within that 2-min segment. The correspondence of areas of high fish abundance along and between transects suggests that the observations cannot be treated as independent. The outlier at about 58°N latitude and 154°W longitude was determined to result from a school of herring.

Changes in depth throughout the strait can affect the observed fish abundance, since the per area observations result from summations over the water column. Consequently, fish abundance per area cannot be considered a stationary process. Relative to fish abundance per area, however, depth changes fairly smoothly over the survey region. If a relationship between depth and fish density (number per square metre per 10-m depth interval) can be found and good estimates of depth over a region can be obtained, then depth may be used as a general predictor of the trend in fish abundance over the survey area, thus accounting for one of the factors contributing to increased variability in fish abundance.

A fish density–depth relationship was estimated empirically from the data (Fig. 2). Fish density was averaged over the entire survey, for each of the 29 depth intervals, with depth intervals below the bottom depth excluded. For this set of observations, fish density increased with depth (Fig. 2). Removing this trend, and calculating the residual fish density averaged over the water column, we are left with 2-min segments of detrended average fish densities (Fig. 3). Each point in Fig. 3 represents the average residual fish density per 10-m depth interval. The observations, detrended by depth, were examined further for an association with changes in the date of the survey or with the observed time of day, but neither of these factors appears to be significantly associated with changes in the residual fish density. The trend in fish density was therefore modeled only as a function of depth.

Spatial Correlation

To examine the spatial correlation between mean fish densities, a subsection of the survey was examined (see Fig. 3). A subsection, rather than the entire survey, was used to reduce the number of computations needed in the analyses discussed later, but was also done to reduce the spatial dimensions of the plot. The section that was used was chosen because it represented a geographically and biologically distinct unit with reference to the strait (i.e. adults were more likely to be found in the center part of the strait, while juveniles were more likely to be found in the southern part). The technique applied here should apply similarly to the other regions outside of the examined subsection as well as to surveys from other years. The subsection examined here consists of 492 locations sampled over a range of longitudes from 154.0 to 155.6°W and a range of latitudes from 57.3 to 58.0°N. The observations were also broken down by depth into an upper layer (intervals 1–17) and a lower layer (intervals 18–29) as discussed later in the analysis. The detrended survey densities of the lower layer are presented on a reduced scale (Fig. 4).

Several approaches may be taken to examine the effect of spatial location on fish abundance. For example, stratifying the survey area and estimating a mean abundance for each stratum is one approach. Examining a contour map of fish abundance over the survey area is another. Contour mapping often incorporates an interpolation algorithm which fits planar surfaces to the observed points and then contours around an estimated grid of values. These values are point estimates taken from the surface of the planes, which correspond to a regular grid of locations placed over the survey area.

Both of these approaches treat the changes in abundance with location as if they were due to changes in some underlying deterministic trend. In the data analyses presented here thus far, we have attempted to detrend the data as much as possible with respect to various auxiliary variables, in particular depth, which were observed in conjunction with fish density observations. If the residual observations are considered to be fully detrended, then the remaining variation in fish density could be assumed to result from some spatial stochastic process. This is the approach that will be taken here.

To examine the stochastic variation between densities at various spatial locations, we shall use a measure of the spatial autocorrelation between points. A useful measure of this is given in terms of the semi-variogram, which is defined as one half the expected value of the squared difference between two points a distance h apart:

$$(1) \quad \gamma(h) = \frac{1}{2} E (W_{x+h} - W_x)^2$$

where, for the analyses to follow, W_x is the detrended density at the point x. (Note, the variogram is defined as twice the semi-variogram.) Under the intrinsic hypothesis of a constant mean

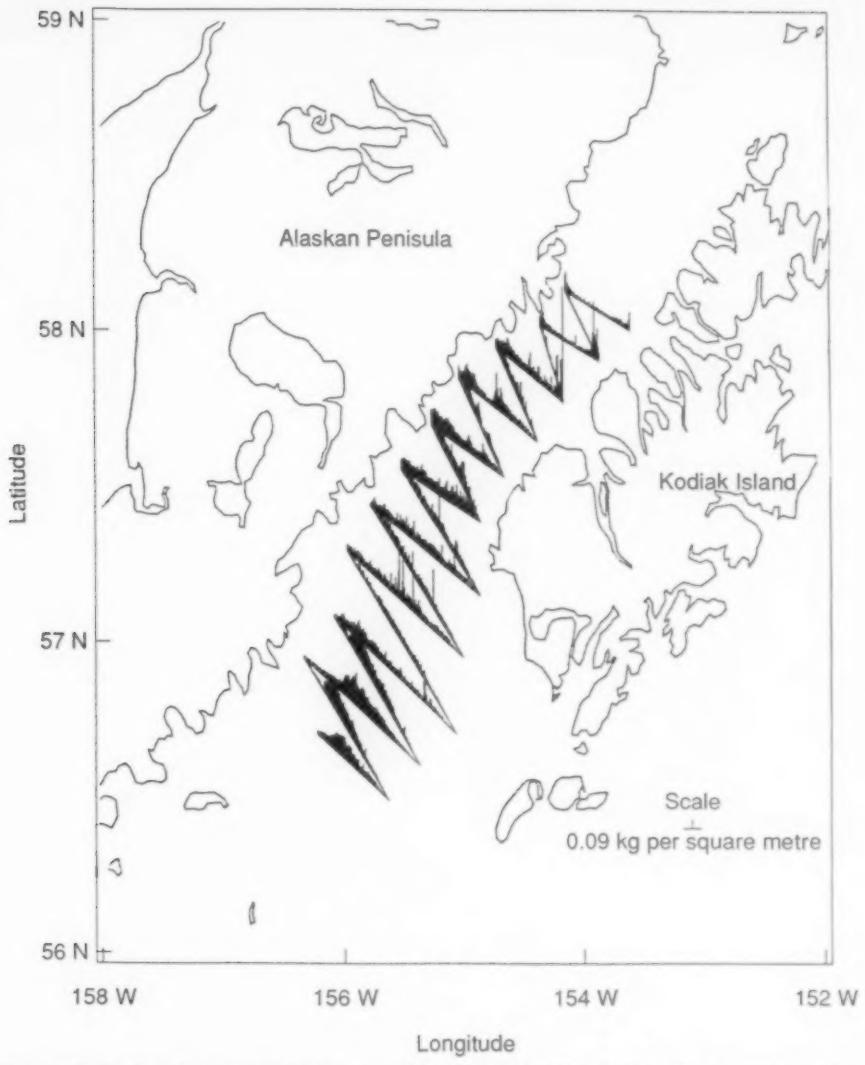

FIG. 1. Shelikof Strait walleye pollock survey of March 25–29, 1987. Fish density summed over the water column per 2-min echo integration output with a total of 1797 observations over 21 transects.

and a finite variance, the difference between two stochastic variables can be expressed as a function of the euclidean distance h between two points x and $x + h$.

When second-order stationarity in the observations can be assumed, the relation between the semi-variogram and the spatial covariance function becomes clearer and can be expressed as:

$$(2) \quad \gamma(h) = C(0) - C(h)$$

where

$$(3) \quad C(h) = \mathrm{Cov}(W_{x+h}, W_x)$$

and $C(0)$ is the a priori variance, or the variance that exists at a point prior to establishing the influence of other points. The semi-variogram, because of its existence under a broader range of assumptions, as well as for its computational convenience, is more often the functional formulation of choice.

To estimate the semi-variogram (effectively the spatial correlation) the difference in density between each pair of observation points in the region is taken. For 492 observations, the result is $\binom{492}{2}$ or 120 786 differences. The traditional estimator of the variogram $2\gamma(h)$ as proposed by Matheron (1963) is

$$(4) \quad 2\hat{\gamma}(h) = \frac{1}{N_h} \sum_{i=1}^{N_h} (W_{t_i+h} - W_{t_i})^2$$

where N_h is the number of observations which are a distance h apart. In practice, a set of indicator distances $\{ h_k : k = 1,2,\ldots\}$ are chosen and the $\binom{N}{2}$ differences are grouped into classes. A difference between two observations is an element of a class

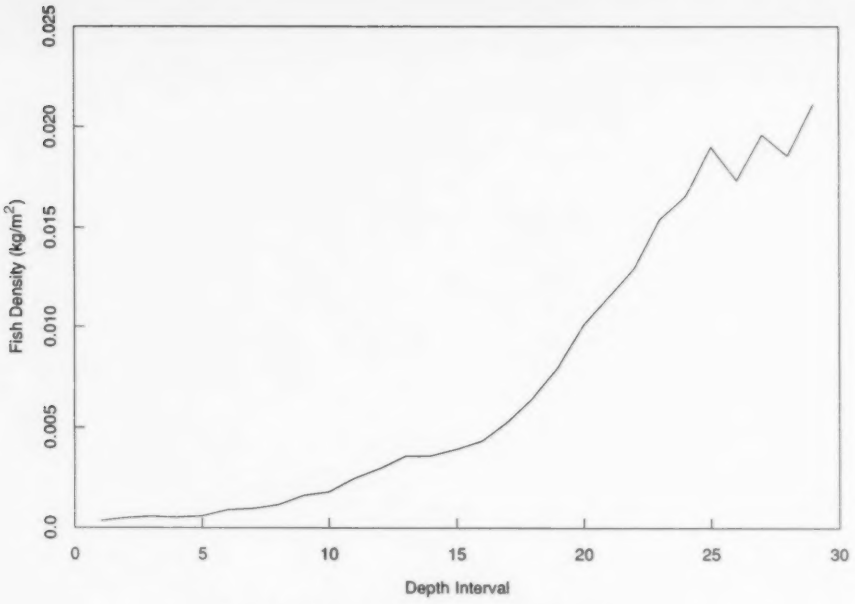

FIG. 2. Fish density – depth interval relationship.

if the distance h between the observations satisfies the constraint ($(k-1)\,d \leq h < (k)d$), where d is a predetermined distance. N_{h_k} is the number of elements in each class and the estimator is applied separately to each class. In order to classify the observation differences according to distance, one must calculate the $\binom{N}{2}$ distances between points and sort the observation differences into classes according to the calculated distances. The calculated distances were based on latitude and longitude locations using the formula for arc distance discussed by Robinson et al. (1978).

Choosing the appropriate number of classes, or equivalently choosing the width d of a class, seems to be something of an art. The number must be chosen such that there are enough classes to depict accurately the form of the variogram model, but too many classes can result in classes which have too few observations. This is particularly a problem at the tails of the range (i.e. for h near zero and for h near the dimensions of the sampling area). Often what is done in practice is that the classes containing a large number of observations, which generally occur in the center of the distance range, are utilized, while those near the dimensions of the sampling area are ignored. The criterion for choosing which classes shall be used and which shall be ignored depends on the number of observations which occur in them relative to the number in the intermediate classes. With hydroacoustic data, some care must be taken in avoiding partitions of the differences which result in a class or classes with few or no observations contained in them over the shorter distance ranges. These ranges can represent the transition between distances taken along a transect versus distances taken between transects. This problem could be exacerbated when using observations taken from surveys with parallel transect lines because of the lack of observations taken at distances shorter than the intertransect distance. There is an even greater risk if the observations are anisotropic, that is if the stochastic relation between points changes with direction. Such a problem can often be detected by contrasting a variogram estimated for one direction with one estimated for another (e.g. across versus along transect lines).

In the analysis to follow, a robust location estimator of the variogram, developed by Cressie and Hawkins (1980), was used:

$$(5)\quad 2\hat{\gamma}(h) = \left(\frac{1}{N_h}\sum_{i=1}^{N_h}|W_{t_i+h}-W_{t_i}|^{1/2}\right)^4/(0.457+0.494/N_h).$$

This estimator is robust to contamination by outliers and is further discussed in Hawkins and Cressie (1984).

The subsection of the Shelikof Strait survey data discussed above was divided into a set representing the upper layer (intervals 1–17, depths ranging from roughly 25 to 185 m) and a set representing the lower layer (depth intervals 18–29, ranging over depths of 195–305 m). This division was for two reasons. First, analyses of earlier surveys suggested this stratification based on observed differences in fish density, and from that standpoint it was a useful demarcation for comparison with earlier methods. Second, subsequent analyses demonstrated that the residual variation was much lower for the upper layer and the observations showed little correlation at the spatial scale of the analysis, suggesting a simple averaging of the data would be the most appropriate approach. In the discussions to follow, only data from the lower layer will be considered.

The graph shown in Fig. 5 shows a plot of the semi-variogram estimates on the lower layer data set versus the midpoint distance (in degrees of latitude) of the associated distances classes. The points are connected by straight lines to facilitate viewing. The variogram tends to increase with distance and eventually reaches a plateau at about half a degree of latitude. The plateau indicates a stationary process on the scale of the area sampled.

Variograms were also computed and compared using latitudinal distances versus longitudinal distances across the sample

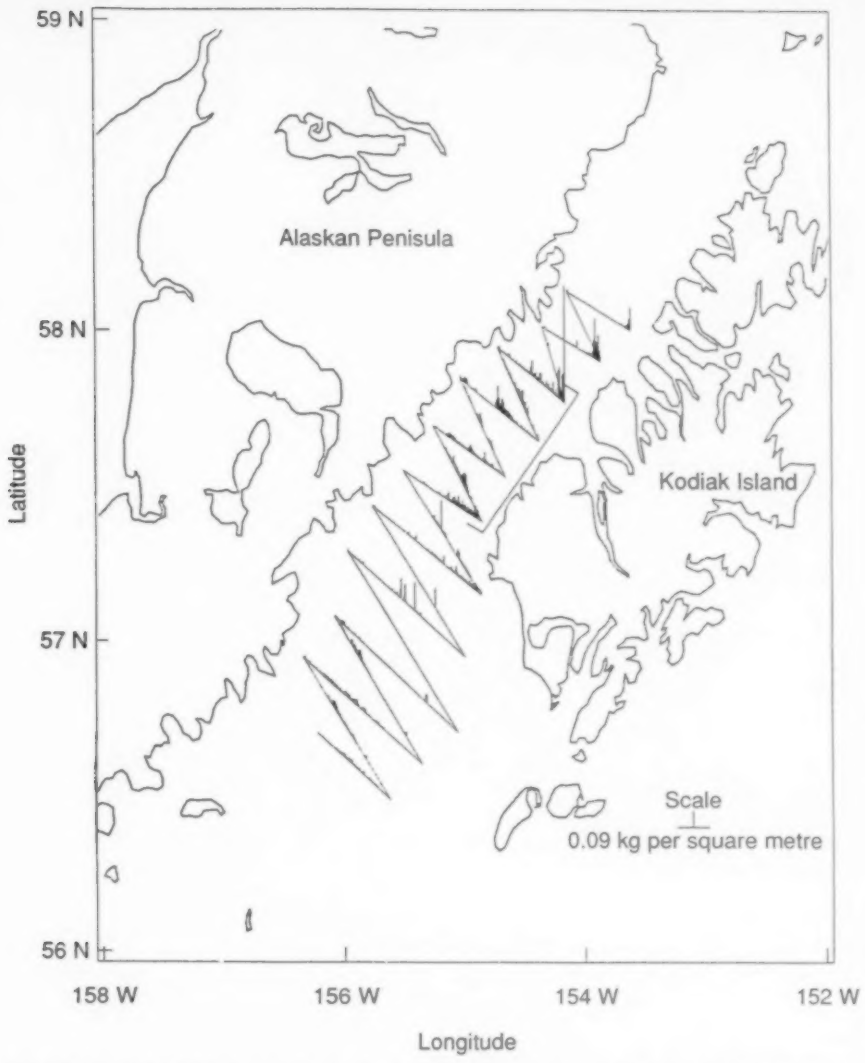

FIG. 3. Mean density per depth interval after removing the density associated with each depth interval. Averages exclude depth intervals below bottom depth. Bracketed area indicates subarea used in the subsequent analysis.

area. No appreciable differences appeared in the distance-dependent correlations with these changes in orientation, and so the process was assumed to be isotropic.

The semi-variogram was modeled using the exponential

$$(6) \quad \gamma(h) = \gamma_1 + (C(0) - \gamma_1)(1 - e^{-h/a})$$

where $\gamma(h)$ reaches 95% of the a priori variance, or still value, $C(0)$ at roughly a distance $h = 3a$ and γ_1 is the *nugget effect* which may be interpreted as the level of variability which exists at distances shorter than the scale of the shortest measured inter-point distance. The nugget effect is a point discontinuity in the variogram at the origin where, by definition, $\gamma(0) = 0$ and $\gamma(h) > \gamma_1$ for $h > \epsilon$ where ϵ is a positive real number. The exponential semi-variogram and other semi-variogram models are extensively discussed, with applications to geological data, in Journel and Huijbregts (1978).

A more familiar perspective perhaps is to examine the covariance function ($C(h) = C(0) - \gamma(h)$) or alternatively the correlation function ($\rho(h) = C(h)/C(0)$, Fig. 6) for the same data set. The correlation drops off exponentially and the observations are virtually uncorrelated at distances greater than about a half a degree latitude.

The variogram may show little change with distance, indicating that there is no apparent stochastic spatial relationship between variables. This can occur when the observations are generated from an independently and identically distributed random process. But it may also ocur when the spatially correlated stochastic process occurs on a different spatial scale from the one on which observations are taken. For example, the observations may be taken too close together or too far apart to detect the coarser or finer scale levels of correlation. These factors must be considered in evaluating the applicability of this procedure.

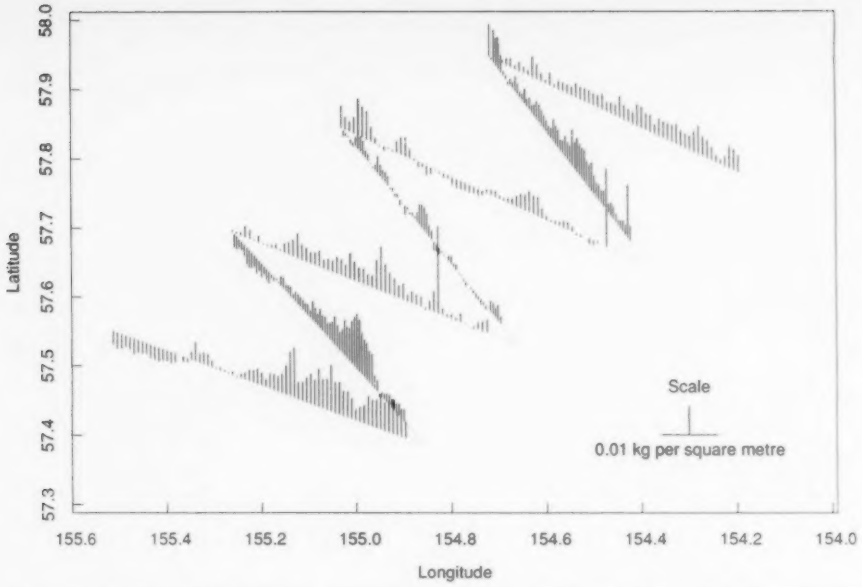

FIG. 4. Reduced set of the detrended mean densities per depth interval (the center seven transects (bracketed) shown in Fig. 3).

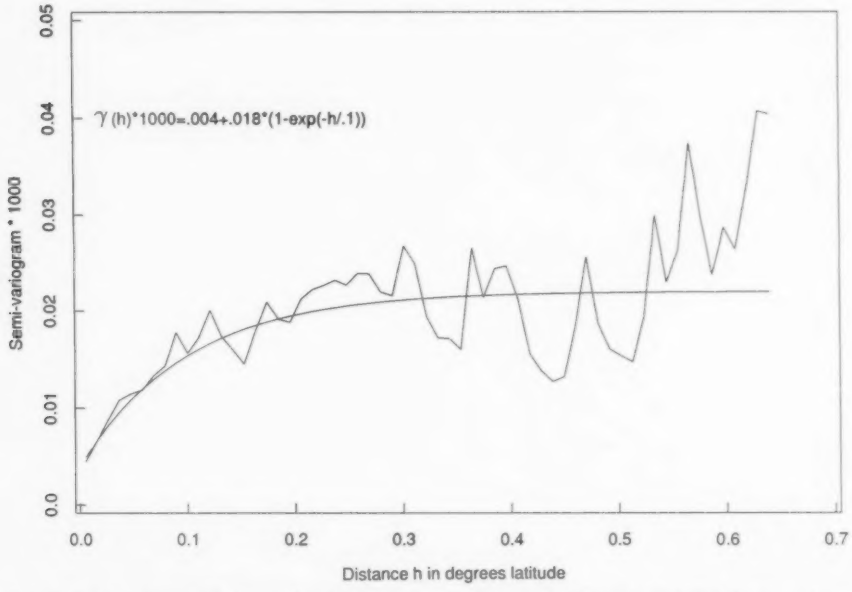

FIG. 5. Semi-variogram estimates for the lower layer (intervals 18–29) on the reduced data set, shown with an exponential model fit (the distance h shown on the x-axis is given in degrees latitude).

Kriging Theory

The theory of regionalized variables, developed by Matheron (1963) and expounded in an applied context by Journel and Huijbregts (1978), was developed for use in the area of geostatistics. The technique uses a known covariance structure, frequently depicted through the variogram, which commonly is a function of the distance between two sample points, to weight the observations taken over a region in order to obtain spatially interpolated, or kriged, estimates of concentrations at any point in the region. The result is an estimate of the concentration, in this case an estimate of fish density, for any point in the region of interest, coupled with the variance of the density estimate. Ripley (1981) gave a theoretical synopsis of the kriging technique and we will use his notation here.

Fish density at a point x in a region (e.g. at a location on a surface or within a volume) will be represented by the random variable Z_x which is made up of a deterministic component μ_x, often called the trend, and a stochastic component ϵ_x as follows:

(7) $Z_x = \mu_x + \epsilon_x$.

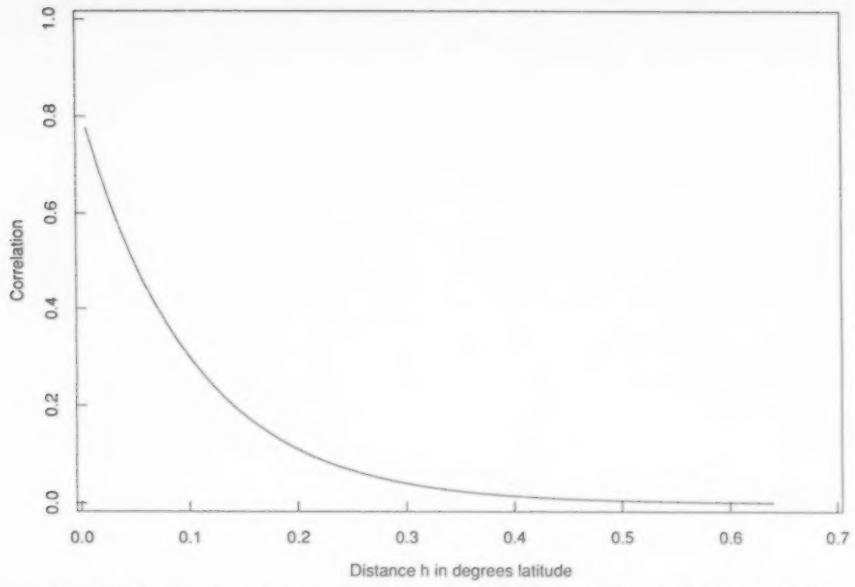

FIG. 6. Correlation function for the exponential fit to the data shown in Fig. 5 (the distance h shown on the x-axis is given in degrees latitude).

Here, trend is interpreted as reflecting changes in fish density which correspond to changes in some environmental condition, like temperature or depth, while the stochastic component is viewed as resulting from small-scale behavioral interactions occurring between the fish themselves. Other interpretations are possible, and through this framework, designs may be set up by which the assumptions upon which the interpretations are based may be tested. If the trend is known and removed, we are left with a zero mean stochastic process W_x where

$$(8) \quad W_x = Z_x - \mu_x.$$

A linear predictor of the detrended density W_{x^*}, at some new point x^* may be formulated as a weighted combination of the process observed at the points $x = x_1, \ldots, x_n$ so that

$$(9) \quad \hat{W}_{x^*} = \sum_i \lambda_i W_{x_i}$$

or, as represented in matrix notation:

$$(10) \quad \hat{W}_{x^*} = \lambda^T \boldsymbol{W}_x$$

where $\hat{W}_{x^*}$ is an estimated single valued function at the single point x^*, $\boldsymbol{W}_x$ is the vector of observations at x, and λ is the vector of weights which is a function of the distances between the new point x^* and the observed points x.

The objective is now to choose a λ which minimizes the squared error

$$(11) \quad E(W_{x^*} - \hat{W}_{x^*})^2 = {\sigma_{x^*}}^2 - 2\lambda^T \boldsymbol{k}_{x^*} + \lambda^T \boldsymbol{K} \lambda$$

where $\boldsymbol{K}$ is the n by n matrix of covariances between the variables occurring at the points x_1 through x_n (i.e. $K_{i,j} = \mathrm{Cov}(W_{x_i}, W_{x_j})$) and $\boldsymbol{k}_{x^*}$ is the n vector of covariances between the n variables at the points x_1 through x_n and the variable at the arbitrarily chosen new point x^* (i.e. $k_{x^*i} = \mathrm{Cov}\,(W_{x^*}, W_{x_i})$). The minimum squared error under the intrinsic hypothesis is achieved when

$$(12) \quad \lambda = \boldsymbol{K}^{-1} \boldsymbol{k}_{x^*}$$

as discussed in Ripley (1981). Substituting (12) into equations (10) and (11), we see that

$$(13) \quad \hat{W}_{x^*} = \boldsymbol{W}_x{}^T \boldsymbol{K}^{-1} \boldsymbol{k}_{x^*}$$

and that

$$(14) \quad \sigma^2_{\mathrm{Error}} = \sigma^2_{x^*} - \boldsymbol{k}^T_{x^*} \boldsymbol{K}^{-1} \boldsymbol{k}_{x^*}.$$

Recall that the covariance function, or equivalently the variogram, is a function of distance alone, so that the matrix and vector functions $\boldsymbol{K}$ and $\boldsymbol{k}_{x^*}$ can now be computed as functions of the distances between the observed points and any other point in the region according to the kriging equations described above.

Kriging Estimates of Abundance

A grid of 100 by 100 points was laid out over a map of the survey region. An estimate was made of the mean and variance in the residual fish density per surface area for each point on the grid using equations (13) and (14). Contour maps showing spatial trends in the means and variances were formulated from these estimates. Figure 7 shows spatially the change in the expected residual fish density, while Fig. 8 shows the change in variance. The pattern in the variance contours reflects the level of confidence in the estimate relative to the location of the transects. The confidence level is higher nearer the transect line where the data are taken and falls off (i.e. the variance gets greater) away from the transect line. To get estimates of surface area density over the area of interest, a measure of depth corresponding to each location is needed (Fig. 9). The grid of depth values was obtained using an interpolation program (Becker et al. 1988) and was based on the depth observations taken with each hydroacoustic observation at each point along a transect. The depth estimates over the region will be treated

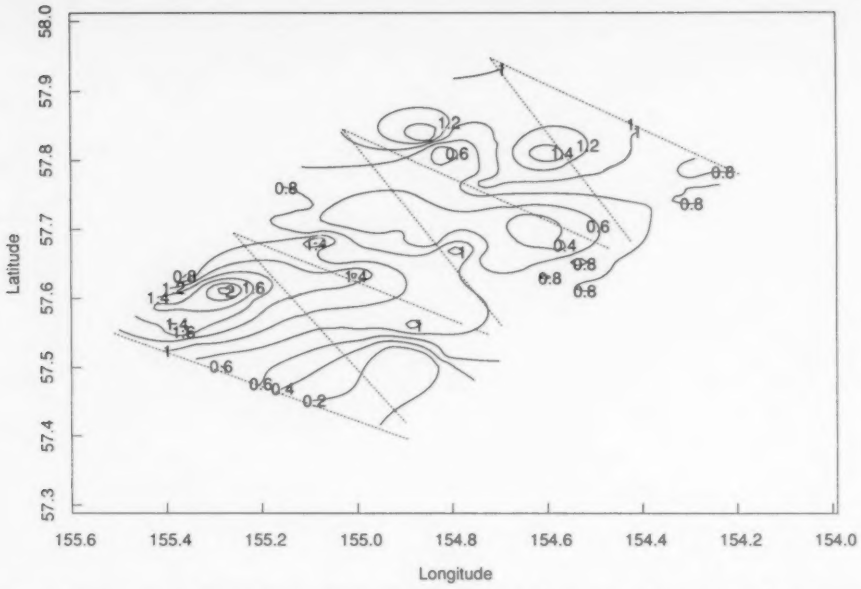

FIG. 7. Spatial mean of detrended density over point grid (scaled by a factor of 100 in kg/m^2). Broken lines indicate survey transects and breaks indicate where no observations were taken.

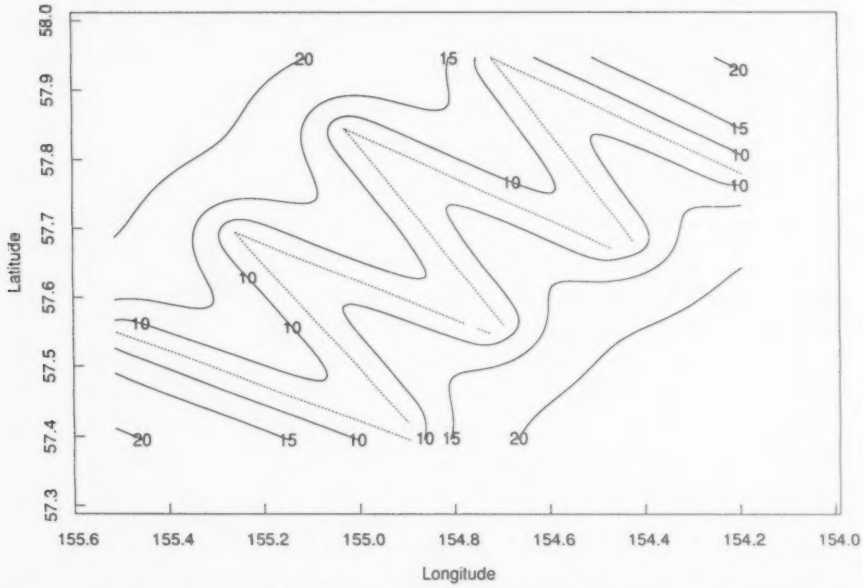

FIG. 8. Spatial variance of detrended density over point grid (scaled by a factor of 10^7). Broken lines indicate survey transects and breaks indicate where no observations were taken.

as if they were known. This assumption is partially justified by comparison of the depth measurements with the density measurements. The depth measurements are much smoother (i.e. have much less variation), and so a smooth contouring of the depth seems reasonable. Depth measurements could also realistically be estimated by other techniques, and through other types of surveys. In each situation, an estimate of the corresponding variance would be useful and could be added to the process of estimating the abundance shown here. For now the variance will be assumed constant.

The density at depth in kilograms per metre squared can now be used to calculate the total biomass in the water column:

$$(15)\quad \hat{Z}_{x^*} = \alpha \sum_{i=1}^{d} (\hat{W}_{x^*} + \mu_{x^*}(i))$$

where i is the depth interval identifier, $d = d_{x^*}$ is the number

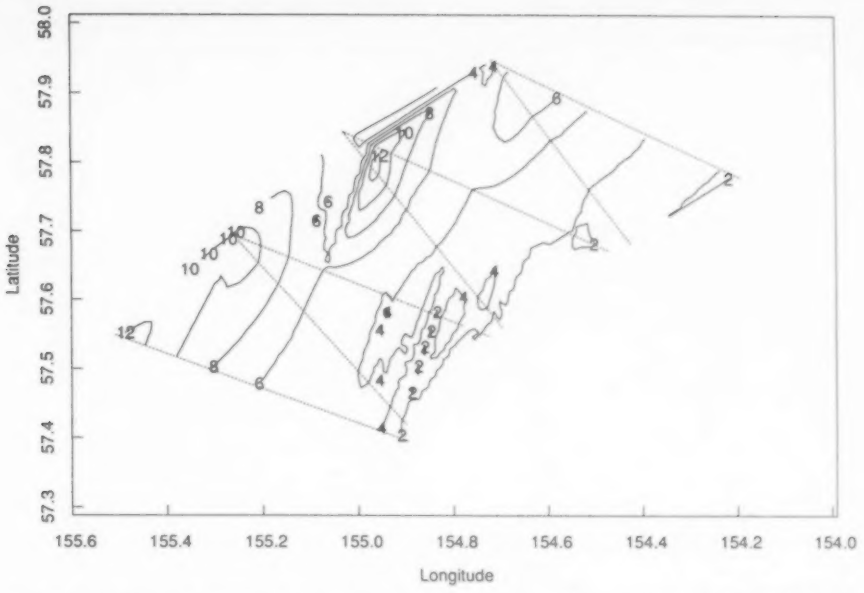

FIG. 9. Depth interpolated from hydroacoustic observations over point grid. Values offset by the minimum depth (184 m) and scaled by a factor of 10^{-1}. Broken lines indicate survey transects and breaks indicate where no observations were taken.

of depth intervals at location x^*, and α is the area represented by each point on the grid. The biomass of fish in the water column at any given observation point x^* is the area times the sum over all depth intervals of the kriged residual mean density per depth interval plus the average density per depth interval. The latter density is obtained from the density–depth relationship (Fig. 2). Equation (15) is the recombining of the trend with the stochastic component as represented by equation (7), except that here the mean is represented as being depth dependent.

To briefly summarize, the average fish density at each depth interval is estimated. At each point over the two-dimensional geographic region, the known depth is used to determine the number of depth intervals at that location. Summing the depth-dependent trend in fish density over those depth intervals and multiplying by the area gives an initial estimate of total abundance of fish in the water column at that location. That initial estimate is adjusted by the average variation per depth interval that results from stochastic spatial variation in residual fish density as calculated through the kriging process. Since this represents the average variation per 10-m depth interval, this variation must also be summed over the appropriate number of depth intervals at each location. Thus, in equation (15), we sum the density at depth plus the average variation at depth for each depth interval over the number of depth intervals present at each location x^*.

Under the assumption of a known mean, the spatial variance is given by

$$(16)\quad \sigma^2_{Z_{x^*}} = \alpha^2 d^2_{x^*}\, \sigma^2_{\text{Error}}$$

the squared area times the square of the number of depth intervals times the residual variation.

Figures 10 and 11 show the spatial density estimates of the mean and variance over the region, this time taking the depth effect into account. Depth modifies the appearance of not only the mean density contours, but the variance contours as well. Particularly high variance areas correspond to deeper regions of the survey area (Fig. 11).

We are now prepared to examine the global estimate of abundance over the area of interest (the lower depth intervals of the previously defined subsection of the survey) with its associated estimate of the global variance. There are several approaches which can be taken. Journel and Huijbregts (1978) discussed several algorithms which, given a set of observations and the variogram, can be used to integrate over an area to achieve estimates of the global mean and variance, or equivalently in the context of a fish population total abundance and the variance in abundance for a specified area. Here we will approximate this integration procedure by using the grid of points over the survey area discussed earlier and summing the values associated with each point over the entire region to estimate the total abundance. The global estimate of abundance for a specified area may be calculated according to the following formula:

$$(17)\quad \hat{Z}_{\text{global}} = \sum_{k=1}^{m} \hat{Z}_k$$

with $\hat{Z}_k$ corresponding to the $\hat{Z}_{x^*}$ of equation (15), where k is the new index corresponding to each of the locations x^* and m is the total number of locations, or grid points, placed evenly throughout the area. To determine the variance of this estimate, note that the covariance may be specified as follows for the detrended estimates $\hat{W}_i$ where $i = 1, m$:

$$\begin{aligned}(18)\quad & E\{(W_i - \hat{W}_i)(W_j - \hat{W}_j)\} \\ &= C(W_i, W_j) - \sum_k \lambda^i_k C(W_i, W_{x_k}) \\ &\quad - \sum_k \lambda^i_k C(W_j, W_{x_k}) + \sum_k \lambda^i_k \sum_l \lambda^i_l C(W_{x_k}, W_{x_l}) \\ &= C_{i,j} - \lambda^i \mathbf{k}_i - \lambda^i \mathbf{k}_j + \lambda^i \mathbf{K} \lambda^i \\ &= C_{i,j} - \mathbf{k}_i^T \mathbf{K}^{-1} \mathbf{k}_j\end{aligned}$$

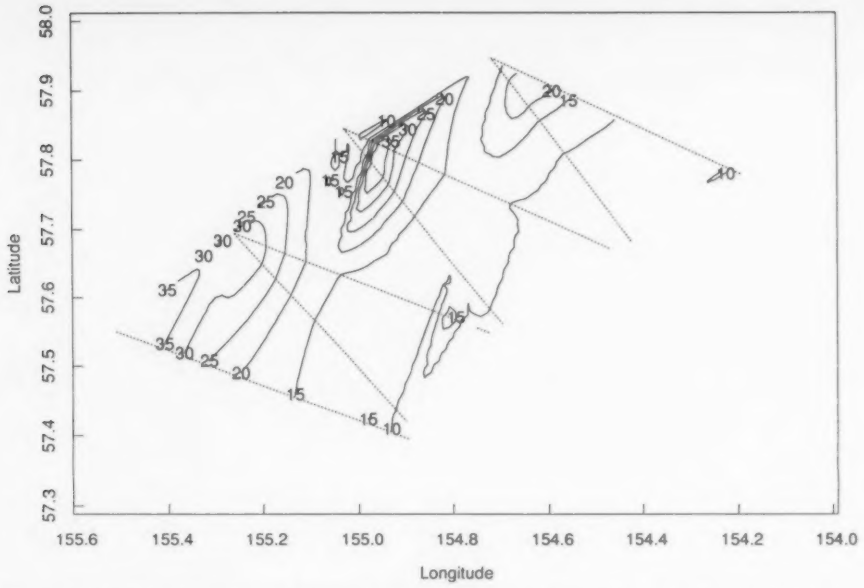

FIG. 10. Spatial mean (scaled by a factor of 100 in kg/m^2) for the lower layer on the reduced data set. Broken lines indicate survey transects and breaks indicate where no observations were taken.

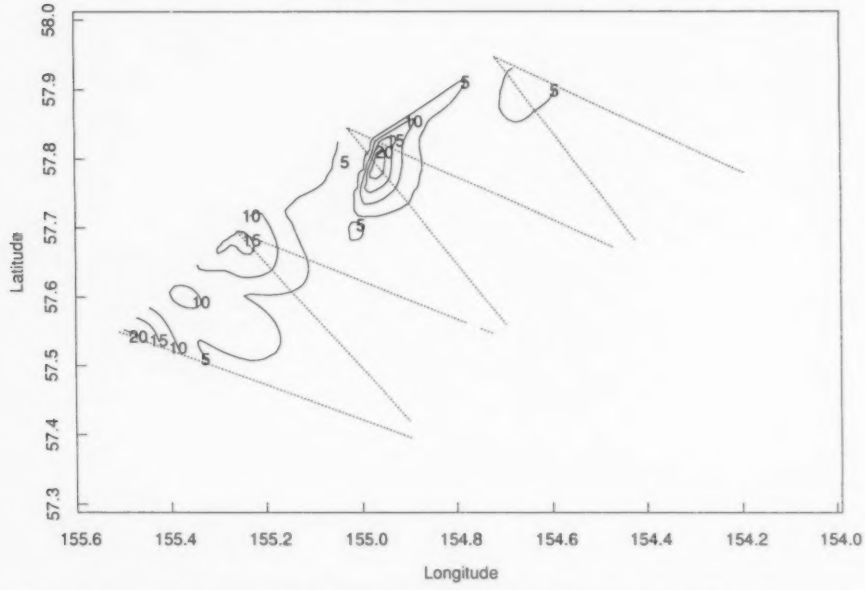

FIG. 11. Spatial variance (scaled by a factor of 10^4) for the lower layer on the reduced data set. Broken lines indicate survey transects and breaks indicate where no observations were taken.

$\lambda^i = \boldsymbol{K}^{-1}\boldsymbol{k}_i$, where λ, $\boldsymbol{k}$, and $\boldsymbol{K}$ are as in equations (11) and (12) with the subscripts k and l of λ corresponding to the observation locations x_k and x_l, respectively, and the subscripts i and j of λ and $\boldsymbol{k}$ corresponding to the location of the estimated points x^*_i and x^*_j, respectively. The estimate of the global variance, based on the detrended predictions, is

$$(19)\quad \sigma^2_{GW} = \sum_{i=1}^{m} \sigma^2_{\text{Error}} + \sum_{i=1}^{m} \sum_{j \neq i} (C_{i,j} - \boldsymbol{k}_i^T \boldsymbol{K}^{-1} \boldsymbol{k}_j).$$

Under the assumption of a known association between trend and depth and again multiplying by the constant α representing the dimension of each subarea, the estimate of the global variance becomes

$$(20)\quad \sigma^2_{GZ} = \alpha^2 \sum_{i=1}^{m} d_i^2 \sigma^2_{\text{Error}} + \alpha^2 \sum_{i=1}^{m} \sum_{j \neq i} d_i d_j (C_{i,j} - \boldsymbol{k}_i^T \boldsymbol{K}^{-1} \boldsymbol{k}_j)$$

where d_i is the number of depth intervals at location i.

TABLE 1. Lower layer total abundance estimates (Mt).

	Number of strata	Total abundance	Variance
Detrended and Kriged		1.95×10^5	1.34×10^7
Stratified transects	1	1.63×10^5	2.61×10^8
Stratified transects	2	1.67×10^5	1.41×10^8
Stratified transects	4	1.71×10^5	5.54×10^7
Stratified transects	6	1.71×10^5	4.63×10^7
Stratified transects	12	1.77×10^5	2.77×10^7
Stratified	12	1.78×10^5	3.49×10^6

Table 1 presents an estimate of total abundance with its estimated variance based on the fitted depth-dependent trend and the kriged residuals. This estimate is compared with estimates made for the same region using stratified systematic sampling approaches classified according to the degree of depth stratification and whether or not transect means are used as single observations. All the estimates shown in Table 1 reflect the lower layer of the subarea shown in Fig. 4.

The five stratified transect approaches (Table 1) calculate the mean fish density by depth strata along each transect and treat the mean as if it were a single observation. In the first example discussed, there are seven transects and a single depth stratum resulting in seven observations going into the estimate. In the second example, there are two depth strata for each of the seven transects resulting in 14 observations. There are 28 observations in the four-stratum calculation and so on. The maximum number of strata is 12 corresponding to all twelve 10-m depth intervals available so that this level of stratification corresponds to the fully depth-detrended approach discussed in the kriged example. The resulting mean μ_i for each stratum i is then computed as

$$\mu_i = \sum_{j=1}^{n} \mu_{i,j}$$

where $\mu_{i,j}$ is the mean density for stratum i and transect j and n is the number of transects. The variance in the mean for each stratum is

$$\sigma_i^2 = \frac{1}{n(n-1)} \sum_{j=1}^{n} (\mu_{i,j} - \mu_i)^2.$$

Each observation used in the stratified transect examples represents the mean density per area and is based on the sum of the densities over the water column within a stratum.

The contoured depth information used in the kriged example was used in the stratified transect examples to determine the total area occupied by a given strata in a manner comparable with what is done in the kriged example. The number of grid points to be included within a stratum i is

$$m_i = \sum_{k=1}^{m} I(d_k \geq d_i)$$

where m is the total number of grid points used in the kriged example and I ( ) is an indicator function that equals 1 when the depth at a grid point d_k is greater than or equal to the depth d_i of the stratum being represented and zero otherwise.

The total abundance in each strata is found by multiplying the total number of grid points m_i for each stratum i by the dimensions of the standard subarea α and the stratum mean μ_i:

$$\hat{Z}_i = \alpha\, m_i\, \mu_i.$$

The variance in total abundance by strata is

$$\sigma_{Z_i}^2 = \sigma^2\, m_i^2\, \sigma_i^2.$$

The estimate of total abundance and the variance in total abundance then follow

$$\hat{Z} = \sum_{i=1}^{s} \hat{Z}_i$$

and

$$\sigma_Z^2 = \sum_{i=1}^{s} \sigma_{Z_i}^2$$

where s equals the number of strata used in the estimate.

The final estimate, the stratified estimate (Table 1), treats all the observations within a stratum as if they are independent observations of the fish density in that stratum. A mean and a variance for each stratum are calculated as before:

$$\mu_i = \sum_{j=1}^{n_i} \mu_{i,j}$$

but now, $\mu_{i,j}$ represents a single per area observation, that is a per area fish density summed over the water column within a stratum, and n_i is the number of observations per stratum. The variance in the mean for each stratum is

$$\sigma_i^2 = \frac{1}{n_i(n_i-1)} \sum_{j=1}^{n_i} (\mu_{i,j} - \mu_i)^2.$$

The estimated variance in this last estimate is greatly reduced because the effective number of observations per strata n_i increases by a factor of 10 or more.

These alternative estimates can be compared with those obtained from the kriged example which uses depth-dependent trends and accounts for spatial correlation. Notable in the comparison of the two sets of estimates is that the kriged estimate of abundance is not substantially different from those arrived at by the stratified methods, yet the estimated variance is quite different. The difference in the total abundance estimates is most likely the result of the way in which the observations are weighted in the estimates. The stratified approaches treat the population as if they were a homogeneous unit over the area of interest whereas the kriged estimates can make greater use of the spatial information present by accounting for correlations that exist between observations over the area.

The variance in the kriged estimate is lower than the variances derived for the stratified transect estimates for two reasons. It is lower primarily because a more appropriate assessment is made of the information available. For the approaches that treat each transect as a single observation, only one degree of freedom is made available for each transect when in fact there may be 20 or 30 observations present along a transect. Since these observations are correlated, the actual degrees of freedom available is somewhat less than the total number of observations, but the kriging approach adjusts for this. The second reason the variance is reduced is that some of the variation in space is being modeled as a stochastic process. In this application the variation in space of the detrended densities is assumed to result from the behavior of the population and not the sampling process. The variance is therefore reduced and expresses only the variation that results from the error associated with sampling.

The variance from the kriged estimate is greater than the variance from the final stratified estimate because the later esti-

mate assumes too many degrees of freedom by not accounting for correlation between adjacent observations.

Discussion

Analysis of the 1987 Shelikof Strait hydroacoustic survey indicates several ways in which estimates of abundance can be obtained while accounting for depth-dependent trends in fish density. By treating changes in density associated with changes in depth as trend, rather than as variation, variances in area-wide estimates of fish abundance can be reduced.

Spatial mean and variance estimates from detrended observations are presented. The variance contours reflect the confidence levels that result from the distribution of sampling effort. Kriged density estimates are combined with depth-dependent trends to give spatial mean and variance estimates of abundance. These estimates are summed to obtain an estimate of total abundance. The kriged estimate of total abundance is the same order of magnitude as a stratified estimator but with a reduced variance. The kriging approach permits better use of both the depth information and the information present in the correlations between observations. Accounting for this spatial correlation can reduce the variance as well.

The comparison between the kriged spatial approach and the stratified approach should be viewed in the context of design-based versus model-based estimates discussed by Smith (1990). Each situation must be judged to determine the applicability of the procedure in question so that problems of bias and low precision may be avoided. Differentiating between factors contributing to observation error versus those resulting from process error may be an important step to take in the analysis.

The work presented in this report is considered a single step in an effort to achieve better estimates of abundance using data collected from hydroacoustic surveys. There are several ways that these estimates can be further improved (although this does not necessarily mean that the variance can be further decreased).

Better estimates of fish density were obtained by using depth as an auxiliary variable. Since depth is automatically taken with each fish density observation over the survey area, it makes sense to use this information in the estimates of fish abundance. Depth was considered a known constant trend, and the relationship between density and depth was also considered fixed and known throughout the survey region. In particular, depth was calculated over the region using a simple contouring algorithm which linearly interpolated depth over the area based on the sample observations. The contour calculations of depth should also be treated as estimates with variance, although that was not done in this study. The variance associated with such depth estimates would increase all the variance estimates presented here. A better approach might be to relate both density and depth to some other observable factor through an approach such as co-kriging (Journel and Huijbregts 1978).

In this application, depth over the survey area was obtained from a single survey whereas several surveys could be combined to get a better overall estimate of depth over the region. The detrended observations were averaged over depth in order to apply a two-dimensional kriging approach. Correlations in all three dimensions (i.e. including depth) could be used to obtain a three-dimensional kriged estimate. (Note that one could also add a fourth dimension, time, using the changes observed from survey to survey within a given sampling season, but the two-dimensional calculations are already taxing enough computationally.)

Based on the observations made here, a systematic zigzag survey over the region appears to be appropriate, although greater sampling effort should be applied to the deeper regions of the Strait if the density–depth relationship is found to hold from season to season. The zigzag design is preferred to the parallel design because information is obtained on correlations between observations (between transects) over a range of distances. In the parallel design the distances between intertransect observations remains relatively constant, providing less correlation information for longer and short distances. However, consideration to changes in fish behavior and fish distribution from year to year should be made in reference to this sampling design recommendation.

The global variance estimate was approximated over the subarea of each gridpoint. There exist many algorithms for extending a kriged point estimate to a kriged area estimate (e.g. see Journel and Huijbregts 1978). The estimate over the entire region was also approximated by using a grid approximation to a global integration. The quality of this approximation can be examined by varying the mesh level and observing the consistency of the estimate.

Here, as in any analytical approach, statistical or otherwise, the outlined computational steps must not be applied blindly or applied without a sound basis of understanding in the biology and behavior of the species of interest. In this instance, depth was used as an indicator of fish density. Other auxiliary variables might be used such as location, water temperature, plankton abundance, or light intensity. In this analysis, depth was chosen because it was a convenient, available, and biologically meaningful variable that contributed much to explaining the observed spatial variation in abundance. This may not be true for all species, nor will it necessarily be true for the species studied here (pollock) at all times. Care must be taken in extending these results.

Several alternative approaches to estimating fish abundance from a series of observations distributed in space are available, including stratified random sampling and the autoregressive techniques discussed earlier. The application of kriging presented here is one simple way of handling the trend while dealing with the observed spatial correlation. In this instance, the spatial estimates provide some additional insights into the problem at hand. These include identifying the contributions to variation that are related to trend and those that result from the spatial setup of the sampling design. The strength of the rationale for using a more sophisticated spatial approach will vary according to the nature of the problem. It may not be feasible estimating total abundance on nonstationary fish schools that vary in density according to some unknown phenomenon. Nor may it be possible on populations for which the time scale of fish movement is shorter than that of the sampling vessel. It also may not be possible to analyze data from a spatial perspective when the level of resolution of the observations is coarser than that of the underlying process. However, there are situations where it may be reasonably applied and which provide interesting and informative results.

Acknowledgments

I thank J. Traynor for his support of this work. I thank E. Nunnallee, N. Williamson, and W. Karp for providing data and for many helpful discussions. I acknowledge and thank J. Simpson and R. Kappenman for reviewing early versions of this manuscript and I thank G.

Stefansson and one anonymous reviewer for providing further helpful insights and comments.

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Trophic Interaction and Competition Between Largemouth Bass (*Micropterus salmoides*) and Rainbow Trout (*Oncorhynchus mykiss*) in a Manipulated Lake[1]

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Hodgson, J. R., C. J. Hodgson, and S. M. Brooks. 1991. Trophic interaction and competition between largemouth bass (*Micropterus salmoides*) and rainbow trout (*Oncorhynchus mykiss*) in a manipulated lake. Can. J. Fish. Aquat. Sci. 48: 1704–1712.

We examined the impact of competition between largemouth bass (*Micropterus salmoides*) and rainbow trout (*Oncorhynchus mykiss*) on diet and condition factor of the two species. Data were collected from Paul Lake (a control lake containing only bass) and Peter Lake (an experimental lake to which trout were introduced) (Michigan, USA). We compared diets of 1988 bass and trout in Peter Lake, 1987 and 1988 bass in both Peter and Paul lakes, and 1988 trout and 1987 bass in Peter Lake. Patterns in diet overlap demonstrated with pooled sample methodology could not always be matched with a random individual pairing technique. With the pooled methodologies, we demonstrated a diet composition shift with significant changes in diet diversity and evenness in the Peter Lake bass after the introduction of trout: such bass ate fewer zooplankton (*Daphnia* spp.) and more odonate naiads than before trout introduction, and their condition factor was reduced. Seasonal divergence in diets between bass and trout was observed. Growth rates of Paul Lake bass did not differ significantly between years, and relative to the experimental lake there were fewer statistically significant dietary differences.

Les auteurs ont étudié l'incidence de la compétition entre l'achigan à grande bouche (*Micropterus salmoides*) et la truite arc-en-ciel (*Oncorhynchus mykiss*) sur le régime alimentaire et le facteur de condition des deux espèces. Des données ont été recueillies dans le lac Paul (un lac de référence n'abritant que des achigans) et dans le lac Peter (un lac expérimental ensemencé de truites) de l'État du Michigan. Les régimes alimentaires ont été comparés comme suit: l'achigan et la truite du lac Peter en 1988, l'achigan des lacs Peter et Paul en 1987 et 1988, et l'achigan et la truite du lac Peter en 1987 et 1988, respectivement. Les régimes de chevauchement du régime alimentaire révélés par l'analyse des échantillons regroupés n'ont pas toujours pu être appariés selon la méthode aléatoire des paires individuelles. Par l'entremise des analyses des échantillons regroupés, les auteurs démontrent une variation du régime alimentaire accompagnée d'importantes modifications de la diversité et de la constance des proies consommées chez l'achigan du lac Peter après le déversement de truites. Ces achigans ont consommé moins de zooplancton (*Daphnia* spp.) et plus de naïades d'Odonates qu'avant le déversement de truites; de plus, leur facteur de condition était moindre. Les auteurs ont aussi observé une différence saisonnière du régime alimentaire entre l'achigan et la truite. Les taux de croissance de l'achigan du lac Paul n'étaient pas nettement différents d'une année à l'autre. Par rapport au lac expérimental, il existait moins de différences statistiquement significatives du régime alimentaire.

Received August 14, 1990
Accepted March 13, 1991
(JA685)

Reçu le 14 août 1990
Accepté le 13 mars 1991

Recently, much attention has been directed toward competitive trophic interactions among fishes (Ross 1986). Most food resource partitioning studies in northern temperate lakes have concerned interactions between closely related species (e.g. Werner 1979; Nilsson and Northcote 1981; Mittlebach 1984; Hume and Northcote 1985; Cochran et al. 1988). Other studies were complicated because they involved multispecies systems (MacLean and Magnuson 1977; Keast 1978; Crowder et al. 1981). Magnan and FitzGerald (1982) studied isolated pairs of nonrelated species, while Nilsson (1963), Andrusak and Northcote (1971), Nilsson and Northcote (1981), and Hume and Northcote (1985) used a control lake to monitor interspecific relationships of competing species.

Our objectives were to describe the effects of interspecific competition on diet and growth rate of largemouth bass (*Micropterus salmoides*), and to compare some niche (diet) overlap methodologies. Largemough bass and rainbow trout (*Oncorhynchus mykiss*) are common species in the upper Midwest including Michigan's Upper Peninsula (Scott and Crossman 1973; Becker 1983), but they generally do not compete for food because of habitat differences. With a 1988 introduction of trout into Peter Lake, we established syntopic populations of comparably sized cohorts of bass and trout where previously only bass were present. In earlier studies, Hodgson and Kitchell (1987), Hodgson and Cochran (1988), and Hodgson et al. (1989) reported that in the absence of interspecific competition, both juvenile and adult largemough bass of Peter Lake were generalist foragers (including zooplanktivory). Trout are generally considered more efficient planktivores than largemouth bass (Becker 1983, and the references therein). Johnson and Hasler (1954) and Kitchell and Kitchell (1980) reported that *Daphnia* spp. were a conspicuous diet component of intro-

[1]Contribution from the University of Notre Dame Environmental Research Center.

duced rainbow trout of Peter Lake in the past. These trout later became extinct because of a lack of suitable breeding substrate. We hypothesized that, upon the reintroduction into Peter Lake, trout would become the predominant zooplanktivore and force the bass into a more generalized and diversified diet. Furthermore, we hypothesized that interspecific competition would be reflected in decreased condition factor (growth rate) of bass.

Methods

Study Lakes

Peter Lake (2.6 ha) and Paul Lake (1.6 ha) are small, humic-stained, seepage lakes located within the University of Notre Dame Environmental Research Center (Section 36, T45N, R42W, Gogebic County, MI). Originally contiguous, the lakes were divided by an earthen dam in 1951 (Johnson and Hasler 1954). Because of a shared watershed, similar morphometries (deep basins with hyperbolic surfaces), and common limnological features (Elser et al. 1986; Leavitt and Carpenter 1989), these nutrient-limited lakes have served as experimental (Peter) and control (Paul) basins as closely as field conditions will permit.

The lakes have a long history of manipulation (Leavitt and Carpenter 1989), but since 1978, Paul Lake has contained an unexploited monoculture of largemouth bass whereas Peter Lake has undergone recent food web manipulations (Carpenter et al. 1985, 1987) including the introduction of 3000 ($1154 \cdot ha^{-1}$) rainbow trout (mean total length (TL) = 168 mm, SE = 2.6 mm). At the time of trout introduction (20 May 1988; day 140), Peter Lake was dominated by juvenile bass and a few reproducing adults (<20). Age II+ bass served as the sample cohort in 1987 whereas ages II+ and III+ constituted the sample population in 1988. The monoculture bass population in Paul Lake consisted of age III+ to > V+ individuals (age III+ and IV+ individuals comprised the majority of the diet samples in 1987 and 1988, respectively). Subadult bass density in Peter Lake was estimated to be $238 \cdot ha^{-1}$ (SE = 32) and $198 \cdot ha^{-1}$ (SE = 22) for 1987 and 1988, respectively, and $242 \cdot ha^{-1}$ (SE = 52) and $203 \cdot ha^{-1}$ (SE = 81) for Paul Lake, respectively.

Data Collection

Fish were collected by angling, which proved to be the most efficient means of obtaining sufficient sample sizes (J. R. Hodgson, pers. obs.). With this method, biases toward the collection of fish of certain prey preferences appeared to be minimal (Hodgson and Cochran 1988), e.g. each collection period contained fish with a wide array of prey items (small zooplankton to juvenile fish). Bass were collected in the littoral zone and trout from the metalimnion near midlake at 2- to 3-wk intervals from May to August. Twelve bass stomachs were sampled on each sampling date in 1987, and 24 of each species were collected in 1988. Bass were held in a live well for <1 h until stomachs were flushed (Seaburg 1957). Trout were usually processed and released immediately at point of capture. Stomach flushing proved to be effective in removing all contents (Hodgson and Kitchell 1987). Weight, total length, and age (from scales above the lateral line just behind the head) were obtained from each bass.

Stomach Analyses

Stomachs of 946 largemouth bass and 161 rainbow trout were examined. All items in each stomach were enumerated. Stomach contents were originally sorted into 20 diet categories but were consolidated into 11 categories for most of the statistical procedures described below: zooplankton (*Daphnia pulex, D. rosea, Holopedium gibberum, Polyphemus pediculus*, and copepods), ephemeropterans, *Chaoborus* spp., water mites, chironomids, coleopterans, odonate naiads and adults, trichopterans, fish (young-of-the-year (YOY) largemouth bass), and an "others" category (benthic macroinvertebrates and terrestrial insects). For random pairing of individuals for niche overlap analysis (see below), 20 and 7 categories were used.

An index of absolute importance (IAI) was calculated (George and Hadley 1979) for each prey category as follows:

$$\mathrm{IAI}_a = \%N_a + \%W_a + \mathrm{FO}_a$$

where %N was the percentage of each food type of the total number of food items, $\%W$ was the percentage weight (milligrams of wet biomass) of each food item of the total weight of foods eaten, and FO was the frequency of occurrence of each food type (the percentage of fish that eat that food type). A summation of all IAI values was then used to calculate the index of relative importance (IRI) for each diet item:

$$\mathrm{IRI}_a = 100 \frac{\mathrm{IAI}_a}{\sum_{a=1}^{n} \mathrm{IAI}_a}$$

where a is the specific food item and n is the number of different food types. The range of IRI for any diet was 0–100.

Statistical Analyses

Relationships among diet parameters were tested with several methods. We compared diets between (1) trout and bass in 1988 in Peter Lake, (2) 1987 and 1988 bass of Peter Lake, (3) 1987 and 1988 bass of Paul Lake, and (4) 1988 trout and 1987 bass of Peter Lake. We expected to document a diet change in Peter Lake bass greater than the interannual changes in Paul Lake. Calculation of the Spearman ranked correlation coefficient (Zar 1984) included 11 prey categories for each sample period even when a particular prey was not consumed. Because each prey was continuously available (J. R. Hodgson and J. F. Kitchell, unpubl. data), the absence or presence of any particular prey category in the diet was considered equally important. Ranked correlation coefficients were calculated with $\%N$, $\%W$, FO, and IRI data. A G-test (using Williams' correction when comparing individual samples and the Bonferoni process when analyzing annual/interannual comparisons; Sokal and Rohlf 1981) calculated on FO was employed as a means of identifying foraging shifts in specific diet categories. Two-tailed Student t-tests of differences in mean IRI values and diet diversity (Shannon–Wiener index; Margalef 1958) of respective paired populations were also employed.

The diet data were also treated with a method which utilized diet overlap comparisons of randomly paired individuals (non-pooled). In an attempt to reduce variability of point estimates of niche overlap methods based on population pooled mean data (e.g. Spearman's ranked correlation coefficient), the data were treated with this alternative pairing test (calculated on $\%W$) as described by Linton et al. (1989). Indices were calculated on 20 diet categories and secondarily on a subset of seven condensed functional categories (zooplankton, odonate naiads, *Chaoborus* spp., chironomid pupae, fish, trichopterans, and others category).

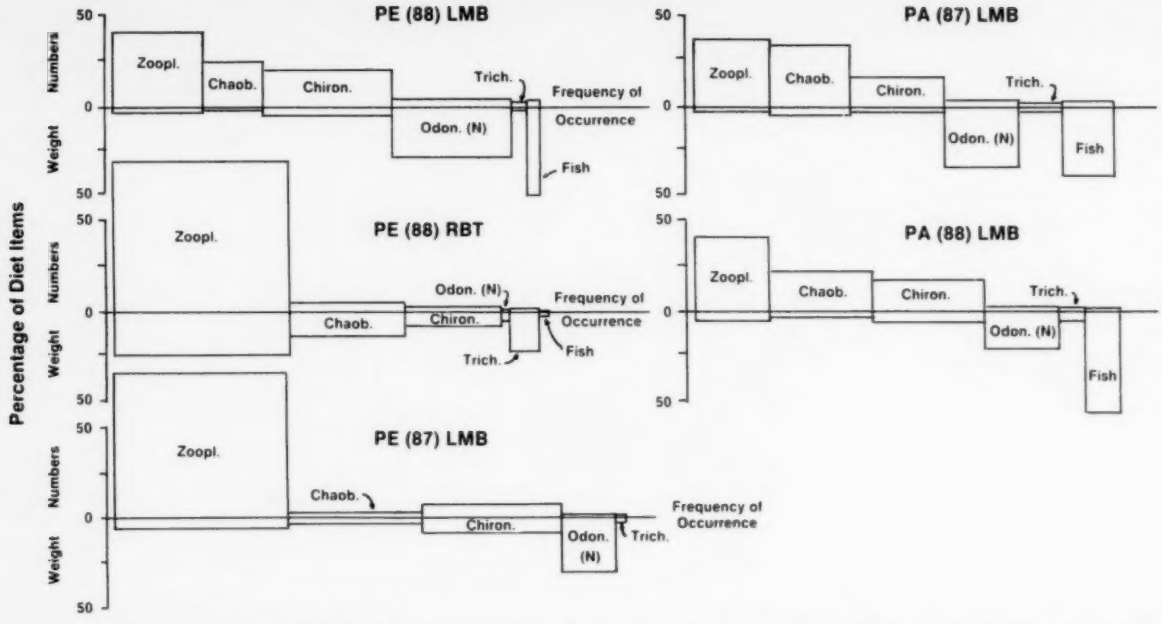

FIG. 1. Percent relative importance (%IRI) of the six most common prey items in largemouth bass (LMB) and rainbow trout (RBT) in Peter (PE) and Paul (PA) lakes for 1987 and 1988. All three statistics necessary to calculate an IRI (%*N*, %*W*, FO; see text for definitions) are graphically represented here. The vertical axis above and below the horizontal axis represent annual %*N* and %*W*, respectively, for each diet category. The horizontal axis (drawn to the same scale as the vertical axes) measures annual FO for each diet category. Note that this can be 100% for any diet category. The rectangular areas that result (three axes) represent respective %IRI of each prey group.

Results

Interannual (1987–88) diet shifts of the magnitude observed for in Peter Lake were not recorded for Paul Lake, and diet differences between bass and trout were evident in Peter Lake in 1988 (see Fig. 1 for descriptive comparisons and Fig. 2 for a statistical treatment). Specifically, the %IRI of zooplankton in trout was significantly greater than in bass whereas the %IRI for chironomid pupae and odonate naiads was significantly greater in bass. With respect to the interannual comparison of Peter Lake bass, zooplankton were more important in 1987 and odonate naiads and chironomid pupae more important in 1988. The 1987 Peter Lake bass diet was more similar to that of 1988 trout than to 1988 bass. Also, starting with sample day 140, 1988, where both bass and trout diets were very similar (Fig. 3), there was a divergence of bass and trout diets. Specific results follow.

1987–88 Paul Lake Bass Comparison

The magnitude of interannual diet variation for Paul Lake bass was less than that recorded for Peter Lake (Fig. 1 and 2). With the possible exception of zooplankton which were slightly more frequent (*G*-value) in the 1987 diet, no apparent interannual trends in FO were evident (only 31.8% of all diet parameter comparisons were significantly different; Table 1). Interannual diets were strongly correlated. A majority (68.5%) of the Spearman's coefficients were significant (see Fig. 3 for %IRI only). But the individual random pairing niche overlap technique (Linton et al. 1989) demonstrated little diet similarity (mean seasonal niche overlap for 20 diet categories = 0.257 (Fig. 4) and 0.330 for 7 categories). Also, interannual diet diversity and evenness (equitability in proportional abundance) were not significantly different (t = 0.61 and 0.17, respectively; Fig. 5 and 6).

1988 Peter Lake Bass–Trout Comparison

Trout ate more zooplankton than bass whereas certain benthic macroinvertebrates were more important in the bass diet (Fig. 1). Specifically, the mean %IRI of *Daphnia* spp. was significantly greater for trout (t = 10.1, $P < 0.0001$) whereas %IRI for chironomid pupae (t = 3.4, $P < 0.01$) and odonate naiads (t = 5.8, $P < 0.001$) was significantly greater for bass (Fig. 2). Zooplankton, *Chaoborus* spp., and water mites occurred significantly more frequently in the trout diet. Odonate naiads were significantly more frequent in the bass diet. Similarly, differences in FO were demonstrated (exclusive of starting date, day 140; 48.5% of the *G*-tests were significant; Table 1).

Bass and trout diets were weakly correlated (see Fig. 3 for %IRI correlations only). For example, only 21.4% of the diet comparisons (r_s calculated on all diet measures) were correlated, and with the elimination of sample day 140, the number declined to 10.7%. Similarly, the Linton et al. (1989) method of niche overlap analysis (Fig. 4) demonstrated minimal diet similarity (mean = 0.235 and 0.184 for 20 and 7 diet categories, respectively). Bass diet was significantly more diverse and even (t = 5.57 and 5.49, respectively, $P < 0.05$; Fig. 5 and 6) than the diet of trout.

1987–88 Peter Lake Bass Comparison

Zooplankton were more important in 1987 (t = 6.9, $P < 0.001$), with chironomid pupae (t = 2.8, $P < 0.04$) and

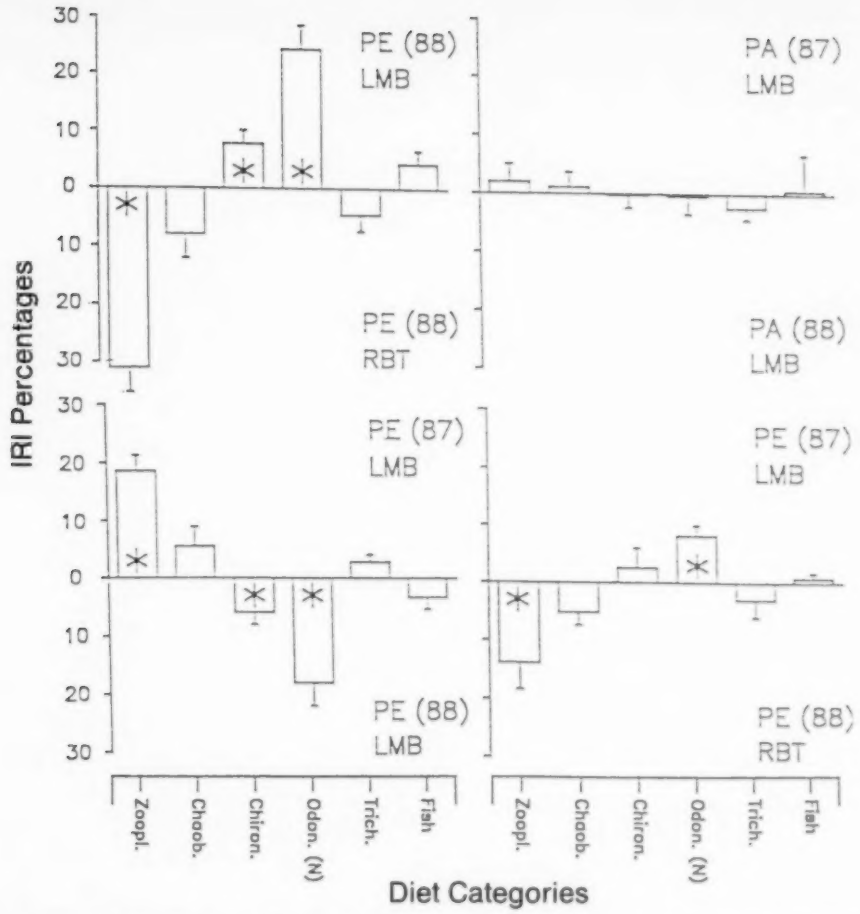

FIG. 2. Mean difference in relative importance (IRI) of the six most common diet items in Peter (PE) and Paul (PA) lakes in 1987 and 1988. Bars above or below the horizontal (zero) line indicate respectively the mean difference of each prey item for the represented fish population (above or below the line). Error bars represent 1 SE and the asterisks indicate a significant difference ($P < 0.05$). Zoopl. = zooplankton, Chaob. = *Chaoborus* spp., Chiron. = chironomid pupae, Odon. (N) = odonate naiads, Trich. = trichopterans, and Fish = juvenile largemouth bass.

odonate naiads ($t = 4.6$, $P < 0.006$) becoming more important in 1988 (Fig. 1 and 2). Significant interannual differences in the frequency of occurrence of diet categories were recorded (52.7% of the *G*-tests, exclusive of day 140; Table 1). Zooplankton and *Chaoborus* spp. had a higher dietary frequency in 1987 whereas odonate naiads were more important in 1988.

Diets were moderately correlated, with only 41.7% of the comparisons statistically significant (r_s of all diet measures; see Fig. 3 for %IRI). With the elimination of day 140, the number of significant correlations declined to 25.0%. Niche overlap analysis (Linton et al. 1989) (Fig. 4; seasonal means = 0.268 and 0.305 for 20 and 7 diet categories, respectively) provided similar results. The diet of the 1988 bass was more diverse than in 1987 (Fig. 5), with mean differences (means of sample periods) in diet diversity and evenness being significantly different ($t = 5.18$ and 7.06, $P < 0.05$, respectively; Fig. 6).

1987 Peter Lake Bass – 1988 Trout Comparison

Diets of 1988 trout were similar to those of 1987 bass (Fig. 1). Zooplankton was the most important diet category of both species (%IRI = 33.8 and 47.1 for bass and trout, respectively) but was significantly more important in the 1988 trout diet ($t = 3.11$, $P < 0.03$; Fig. 2). Chironomid pupae and *Chaoborus* spp. were of similar importance to both species (%IRI ranged from 12.1 to 15.1). Odonate naiads were significantly more important as a bass food ($t = 4.5$, $P < 0.007$).

Ephemeropterans, odonate naiads, and the others diet category were more common in the bass diet. *G*-test comparisons were similar to the Paul Lake bass (Table 1). Only 33.3% of the diet comparisons were significant. Collectively, Spearman's correlation analysis demonstrated a strong diet correlation between bass and trout (Fig. 3). Pooling diet measures, 66.6% of the comparisons were statistically significant. However, niche overlap was not evident with the Linton et al. (1989) analysis (Fig. 4). With the possible exception of the last sample period comparison, diets were not similar (seasonal mear overlap = 0.260 and 0.258 for 20 and 7 diet categories, respectively). There were no significant differences in the mean diet diversity or evenness ($t = 0.56$ and 0.60, respectively; Fig. 5 and 6).

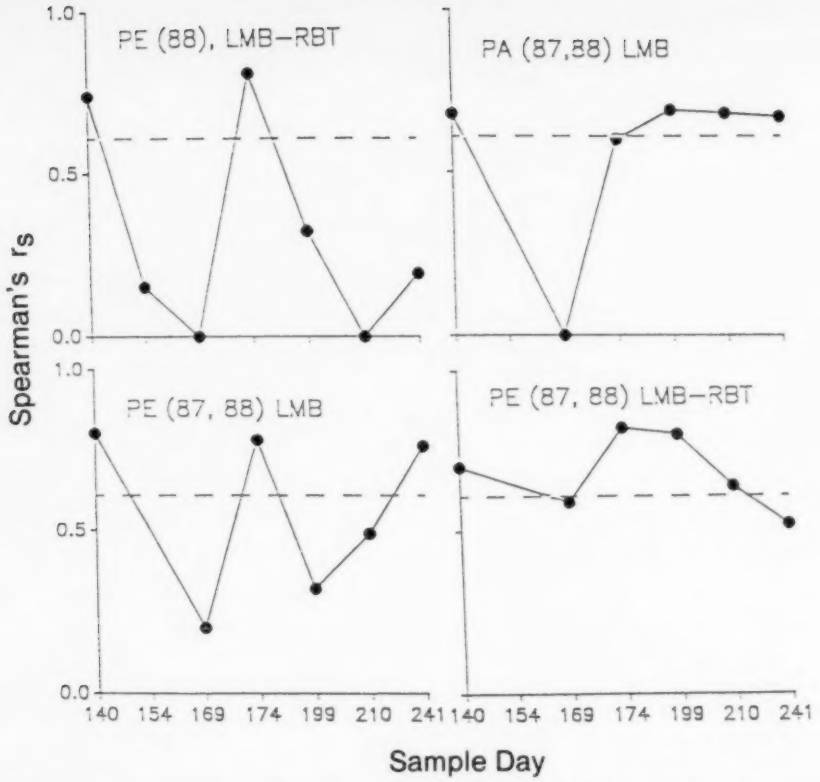

FIG. 3. Spearman's correlation coefficient index (r_s) calculated on indices of percent relative importance (%IRI) for each sample period for largemouth bass (LMB) and rainbow trout (RBT) in Peter (PE) and Paul (PA) lakes in 1987 and 1988. The broken line represents the significance level ($P < 0.05$).

TABLE 1. *G*-test (with Williams correction) calculated on FO of 11 diet categories. Each value represents a significant difference ($P < 0.05$) for that diet item at a particular sample period and respective diet comparison. Diet comparisons: 1 = bass and trout of Peter Lake in 1988, 2 = bass in Peter Lake in 1987–88, 3 = bass in Paul lake in 1987–88, and 4 = bass in Peter Lake in 1987 and trout of Peter lake in 1988. Zoopl. = zooplankton, Ephem. = Ephemeroptera, Chaob. = *Chaoborus* spp., Chiron. = chironomid pupae, Coleo. = adult coleopeterans, Odon. (N) = odonate naiads, Odon. (A) = odonate adults, Trich. = tricopterans.

Diet category	Sample days					
	140	169	179	199	210	241
Zoopl.	- - 3 -	1 2 3 -	1 2 3 -	1 2 - -	1 2 3 -	1 2 - -
Ephem.	1 - 3 4	- - - -	- - - 4	1 - - 4	- - - -	- 2 - 4
Chaob.	1 2 - 4	1 2 - -	1 2 - 4	1 2 3 -	1 2 - -	1 2 - -
Mites	1 - - 4	1 2 3 -	1 2 3 -	1 2 - 4	1 2 - -	1 2 - -
Other	1 - 3 4	- 2 - 4	- - 3 -	- 2 - -	1 - 3 4	- - - 4
Chiron.	- - - -	- - - -	1 - - 4	- - - -	- - - 4	1 - -4
Coleo.	- - - -	1 2 3 -	- - - -	- 2 3 4	1 2 3 -	- - - -
Odon. (N)	1 2 - -	1 - - 4	1 2 - 4	1 2 - 4	1 - 3 4	1 2 - 4
Odon. (A)	- - - -	- 2 3 -	- - - -	- - - -	- - - -	- - - -
Trich.	- 2 3 -	- 2 3 -	- 2 - 4	- - - -	- - - -	1 2 - -
Fish	- - - -	1 2 3 -	- - - -	- - - -	- - 3 -	- 2 3 -

Overall Comparisons

When the 1987–88 Paul Lake bass were compared with the 1988 Peter Lake bass–trout interaction, the Peter Lake comparison had more significant seasonal and individual sample period differences than Paul Lake (G_{adj} = 9.73 and 7.04, $P < 0.05$, respectively). Statistically the same results were generated with the Peter Lake interannual bass contrast and Paul Lake (Table 2). When only manipulated populations were compared, no statistical differences were generated (Table 2), each having about the same number of correlations. For example, a

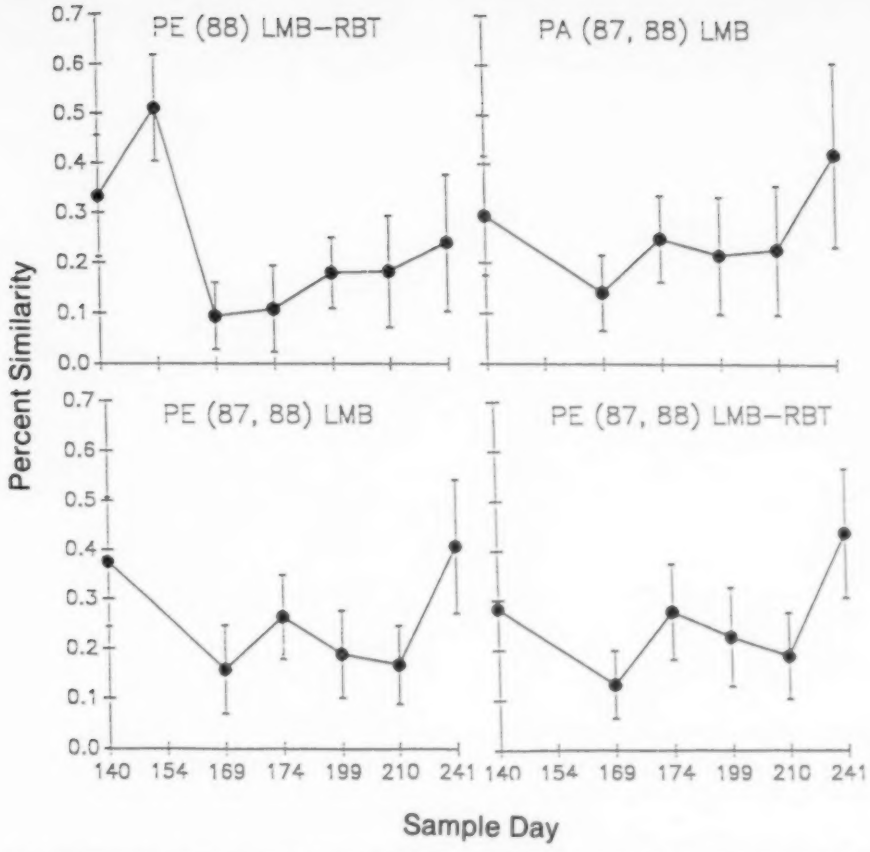

FIG. 4. Percent similarity (Linton et al. 1989) of diets (± 2 SE) for largemouth bass (LMB) and rainbow trout (RBT) for Peter (PE) and Paul (PA) lakes for each sample period in 1987 and 1988. Each graph represents a single niche overlap comparison and it is based on 20 prey categories.

comparison of 1988 Peter Lake bass and trout with interannual comparison of Peter Lake bass showed no difference (G_{adj} = 0.17 and 0.14, $P < 0.05$, respectively, for seasonal and individual sample periods).

A comparison of bass interannual mean condition factor was used as an additional index of interspecific competition. In Peter Lake the slope of the weight–length regression line was significantly greater in 1987 than in 1988 ($F = 23$, $P < 0.001$; Table 3), indicating greater condition factor and suggesting a reduction in growth rate in 1988. There was no interannual significant difference in Paul Lake ($F = -0.19$).

Discussion

We compared diets and growth rates of bass in the presence and absence of trout in a manipulated lake with those of bass in a control lake. Although not all diet comparisons were statistically significant, trends indicated that bass and trout competed. For example, more significant statistical tests (i.e. *G*-tests) were generated from the bass–trout interaction or interannual Peter Lake bass than those of Paul Lake (Tables 1 and 2; Fig. 3). Diets were more correlated (Spearman's ranking) for Paul lake fish between years than for other comparisons. Percent IRI results (Fig. 1 and 2), diet diversity, and evenness comparisons (Fig. 5 and 6) further supported a interspecific interaction. Trout became the predominant zooplanktivore and bass become more generalized in their prey selection.

Earlier diet shifts have been documented for Peter and Paul lakes (Hodgson and Kitchell 1987). In 1981, in response to a reduction in intraspecific competition (partial winterkill), bass became better foraging specialists as reflected in reduced diet diversity. In 1988 the diet of bass (same gene pool as the 1981 population) became more diversified (generalized) in prey selection. With the exception of zooplanktivory, the response in 1988 was the opposite of that which occurred between 1980 and 1981. Diet change associated with increased age is widely known, with younger fish feeding on small prey (i.e. zooplankton) and shifting to larger prey sizes (i.e. benthos) as they become more efficient in capturing and handling larger prey. Similar ontogenetic foraging shifts have been observed here (J. R. Hodgson, unpubl. data) between YOY and age I+ fish. But with older cohorts (ages II+ and III+ or older), diets of Peter and Paul Lake bass are remarkably similar when in monoculture conditions (Hodgson and Kitchell 1987; Hodgson et al. 1989). Age-specific influences are not exclusive in effecting observed diet shifts.

Relationships between form and function are significant to feeding ecology (e.g. Keast and Webb 1966; Gatz 1981). Given largemouth bass mouth/gill raker morphology, the diet shift by bass away from zooplanktivory was expected. Bass are not effi-

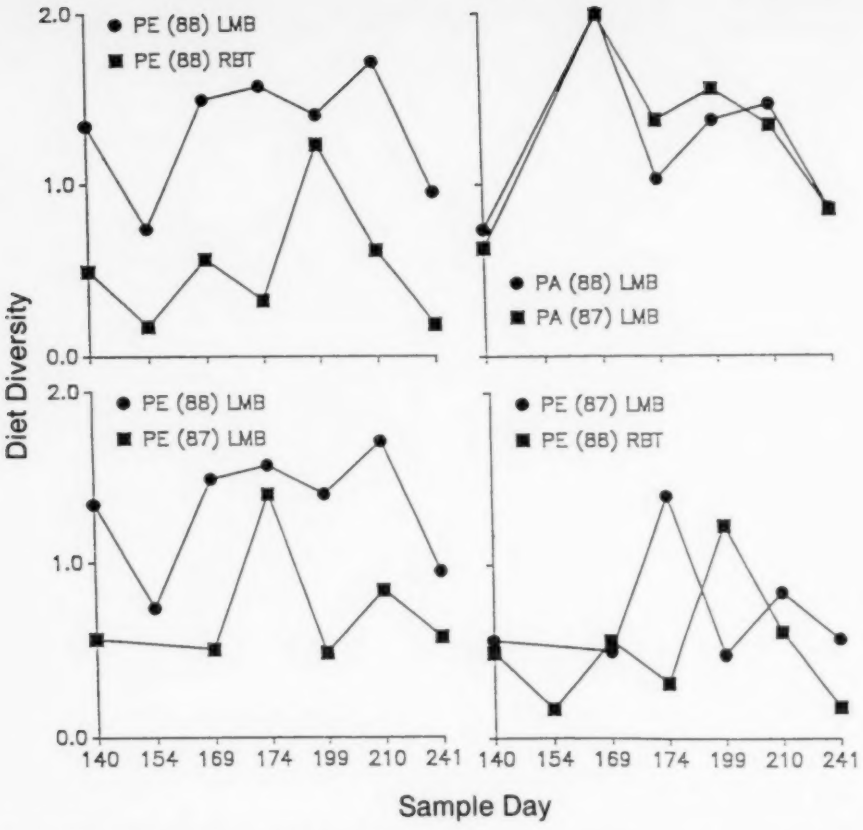

FIG. 5. Diet diversity (Shannon–Wiener index) for each sample period for largemouth bass (LMB) and rainbow trout (RBT) in Peter (PE) and Paul (PA) lakes in 1987 and 1988.

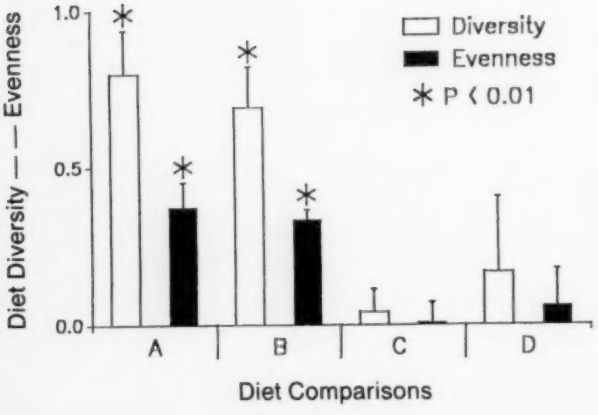

FIG. 6. Mean difference in diet diversity and evenness (prey category proportional equitability) based on individual sample periods. Comparisons: A = 1988 largemouth bass and rainbow trout in Peter Lake, B = 1987 and 1988 largemouth bass in Peter Lake, C = 1987 and 1988 largemouth bass in Paul Lake, and D = 1987 largemouth bass in Peter Lake and 1988 rainbow trout.

cient zooplanktivores, although Hodgson and Kitchell (1987) reported that in the absence of interspecific competition and under intense intraspecific competition, *Daphnia* spp. became an important diet item. Rainbow trout are generally considered more effective zooplanktivores than bass (Becker 1983, and references therein) and have been reported to be effective size-selective predators in Peter Lake (Johnson and Hasler 1954; Kitchell and Kitchell 1980).

In addition, differences in temperature (habitat) preferences of bass and trout probably influenced the relative difference in importance of benthic prey in their diets. Rainbow trout have lower temperature preferences (Garside and Tait 1958) than largemouth bass (Scott and Crossman 1973). In Peter Lake the summer temperatures of the littoral zone exceeded the preferred temperature of trout, thus restricting them to the metalimnion and away from the primary source of odonate naiads and chironomid pupae. Respective prey and predator habitat preferences probably also influenced respective prey selections, but temperature preference was not sufficient cause to prevent bass from foraging on *Daphnia* spp. at midlake. Hodgson et al. (1989) reported that bass were frequently observed at midlake and that they descended to the metalimnion to feed.

We compared niche overlap by using estimates of population sample centroids (pooled data). Linton et al. (1989) identified biases associated with this techniques and recommended using an alternative method which employed randomly paired indi-

TABLE 2. G-statistic (Bonferoni process) calculated on the number of significant G-tests on 11 diet categories for collective seasonal data and for individual sample periods for largemouth bass (LMB) and rainbow trout (RBT) in Peter (PE) and Paul (PA) lakes in 1987 and 1988. $^*P < 0.05$.

Comparison	Seasonal			Individual		
	Significant	Not significant	$G_{adj.}$	Significant	Not significant	$G_{adj.}$
PE LMB 88 – PE RBT 88	6	5		20	57	
PE LMB 87 – PE LBM 88	5	6	0.17	19	47	0.14
PE LMB 87 – PE LMB 88	5	6		19	47	
PE LMB 87 – PE RBT 88	3	8	0.74	22	44	0.31
PE LMB 88 – PE RBT 88	6	5		20	57	
PA LMB 87 – PA LMB 88	0	11	9.73*	6	60	7.04*
PE LMB 87 – PE LMB 88	5	6		19	47	
PA LMB 87 – PA LMB 88	0	11	7.62*	6	60	8.51*

TABLE 3. Weight–length regressions of largemouth bass of Paul and Peter lakes for 1987 and 1988. $^*F = 23$, $P < 0.001$.

Lake and year	n	Regression [$\log Y = a(\log X) + b$]		
		a	b	r^2
Peter				
1987	219	−12.8	3.27	0.95
1988	230	−11.0	2.92*	0.91
Paul				
1987	220	−11.3	3.00	0.97
1988	155	−11.9	3.10	0.95

viduals (nonpooled data). We also used this method, but obtained comparable results (two methods) only when comparing Peter Lake bass and trout diets in 1988 and interannual differences of Peter Lake bass. We did not obtain the hypothesized interannual niche overlap similarity in Paul Lake or between the 1987 Peter Lake bass population and the 1988 trout.

There are several possible explanations for the observed differences. It is possible that the diet comparisons of Paul Lake or the 1987 bass and 1988 trout diets had less diet overlap than our descriptive (Fig. 1) or pooled statistical methods indicated (Tables 1 and 2; Figs 2–6). Other explanations may involve differences in relative abundance and availability of the prey types, or size (biomass) range of prey, or number of prey categories in the two studies. Manly (1989) also reported similar as well as additional concerns with the individual random pairing methodology.

The Linton et al. (1989) study used nine prey types of relatively equal size (eight insect orders and one arachnid) whereas in our study, niche overlap was based on 20 prey categories of a wide range of size (*Daphnia* spp. to juvenile fish). Of these, four of the prey categories (*Daphnia* spp., *Chaoborus* spp., chironomid pupae, and odonate naiads) occurred regularly and abundantly in the diets. The remaining items occurred irregularly with much intrasample (fish-to-fish) variance. Hodgson et al. (1989) reported on the range and importance of diet variance in the Peter Lake bass population. This array of less common items ("dietary tail") may influence niche overlap outcomes. Since each diet category is given equal importance in the niche overlap calculation, overlap (actual similarity) may not be demonstrated if the diet contains a long tail of uncommon, variable diet items. But even with a shortening of the dietary tail with a reduction in prey categories (consolidation into seven functional groups) the results did not change, indicating that the length of a dietary tail in itself may not be sufficient to influence results.

Condition factor (growth rate) of Peter Lake bass significantly declined in 1988 ($P < 0.001$; Table 3) which may be attributed to interspecific competition, but caution is necessary. There was no corresponding reduction in Paul Lake. The addition of trout to Peter Lake increased total predator intensity several times, effecting increased intraspecific competition. Also, differences in age composition need consideration when making interlake or interannual comparisons. As a population ages, growth rates generally slow. With annual recruitment of new individuals (ages III+ and IV+) into Paul Lake samples, the overall age composition remained basically consistent between years. In Peter Lake the population did age between years, but age-specific biases were lessened with the recruitment of age II+ individuals into the 1988 sample. We argue that interannual comparisons were valid and that the reduction in condition factor in Peter Lake was effected more by competition than age-specific influences.

Connell (1983) discussed measures of determining interspecific competition. He suggested that responses relative to resource breadth usage (e.g. changes in microhabitats or food types) or abundance (including factors influencing abundance, e.g. feeding activity, growth, etc.) be measured in conjunction with the altering of abundance of one species. If statistically significant differences occurred between experimental and control populations, interspecific competition could be concluded to occur. On the basis of this type of evidence, we conclude that bass and trout competed for food in Peter Lake.

The use of whole-lake experiments to test competitive interactions between species may prove problematic if the lakes are limnologically dissimilar (Hume and Northcote 1985), or unreplicated (Hurlbert 1984; Likens 1985; Stewart-Oaten and Murdoch 1986; Fausch 1988; Carpenter 1989). Although temporal pseudoreplication was inherent in our experimental design, some of the criticisms are addressed, in part, with the use of a control lake. Expected changes can be explained as a function of the manipulation and should be greater than the variation in the control lake. We demonstrated sufficient effects (Connell 1983) to support the conclusion of interspecific competition.

Comparable methodologies (pooled or nonpooled comparisons) did not always corroborate results (e.g. similarities of

trophic niche overlaps, where hypothesized). The absence of experimental replication with a dependence on temporal pseudoreplication may have further confounded our results and their interpretation. But the use of a control lake with a monoculture population of bass certainly has reduced the limitations of an experimental design without replication. Knowing the strength of variation (i.e. interannual) can help identify the magnitude of change associated with a manipulation.

In the absence of competition, a species may not always be constrained by the limitations of functional morphology. For example, Peter Lake largemouth bass foraged effectively on zooplankton in the absence of competition, but became less effective zooplanktivores when competing with rainbow trout. Divergence in diet did not result exclusively from differences in microhabitat use, but is reflected in behavioral and trophic morphological differences of the two species. These differences are important to understanding possible outcomes where these two species may coexist as competitors in small Midwestern lakes.

Acknowledgements

We are indebted to the personnel of the UNDERC for providing logistical support and to Michael Lang for his assistance in computer graphics. We thank P. A. Cochran and two referees for their comments on the manuscript. Thanks are expressed by S.M.B. to St. Norbert College for its support of undergraduate research and the National Science Foundation for its REU support. The work was supported by National Science Foundation grant BSR 86-04996.

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Mortality and Recruitment of Pacific Sardine, *Sardinops sagax caerulea*, Larvae in the California Current

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Butler, J. L. 1991. Mortality and recruitment of Pacific sardine, *Sardinops sagax caerulea*, larvae in the California Current. Can. J. Fish. Aquat. Sci. 48: 1713–1723.

The mortality rates of Pacific sardine, *Sardinops sagax caerulea*, larvae (<20 d) increased during 1951–67, and this increase was positively correlated with the increase in biomass of northern anchovy, *Engraulis mordax*. Other potential correlates with larval sardine mortality rates were examined using multiple correlation. These included temperature and zooplankton volumes at stations positive for sardine larvae, water column stability, upwelling, transport, salinity anomalies, and Pacific mackerel, *Scomber japonicus*, abundance. None contributed significantly to the multiple regression with anchovy abundance. Thus, no evidence existed for either Lasker's stable ocean hypothesis of larval mortality or Hjort's larval transport hypothesis. The logarithm of the ratio of sardine recruits at age 2 to the spawning biomass was used as an index of recruitment success. Sardine recruitment success at age 2 was not correlated with larval mortality rates. Sardine recruitment, however, was negatively correlated with both northern anchovy biomass and the combined biomass of Pacific sardine, Pacific mackerel, and northern anchovy. A plausible explanation for this result is that predation by pelagic fishes results in greater mortality rates of sardine larvae and juveniles which in turn decrease sardine recruitment.

De 1951 à 1967, les taux de mortalité de larves (< 20d) de sardine du Pacifique (*Sardinops sagax caerulea*) ont montré une augmentation en corrélation positive avec l'augmentation de la biomasse d'anchois du Pacifique (*Engraulis mordax*). L'auteur a utilisé une corrélation multiple pour étudier d'autres facteurs potentiels en corrélation avec les taux de mortalité des larves de sardine, en particulier la température et les volumes de zooplancton présents aux stations fréquentées par des larves de sardine, la stabilité de la colonne d'eau, les remontées d'eau profonde, le transport, les anomalies de la salinité et l'abondance du maquereau (*Scomber japonicus*). Aucun de ces facteurs n'a contribué de façon significative à la régression multiple en fonction de l'abondance de l'anchois. L'hypothèse de l'océan stable de Lasker concernant la mortalité des larves et l'hypothèse du transport des larves de Hjort ne sont donc pas étayées. L'auteur utilise le logarithme du rapport entre le nombre de recrues de sardine de 2 ans et la biomasse de reproducteurs comme indice du succès du recrutement. Le succès du recrutement de sardines de 2 ans n'est pas en corrélation avec les taux de mortalité des larves. Par contre, le recrutement des sardines est en corrélation négative avec la biomasse d'anchois du Pacifique et la biomasse combinée de la sardine, du maquereau et de l'anchois. Une explication plausible de ce résultat serait que la prédation exercée par les poissons pélagiques est la cause de taux de mortalité plus élevés des larves et des juvéniles de sardine, qui ont pour résultat une baisse de son taux de recrutement.

Received May 16, 1988
Accepted March 13, 1991
(J9734)

Reçu le 16 mai 1988
Accepté le 13 mars 1991

The causes of larval fish mortality have been the subject of considerable speculation for several decades. Most speculation is on the relative importance of starvation or predation in determining the mortality rate of larvae.

The starvation hypothesis is based in part on the observation that larval fish reared in the laboratory require greater food densities than are generally found in the ocean. Field and laboratory experiments (Hjort 1913, 1926; Lasker 1975) have supported the idea that the availability of food affects the survival of first-feeding larvae. The incidence of starving larvae of northern anchovy, *Engraulis mordax*, and jack mackerel, *Trachurus symmetricus*, in plankton net collections suggests that starvation mortality is restricted to the first 1–2 wk after yolk sac absorption (O'Connell 1980; Hewitt et al. 1985; Theilacker 1986).

The basis of the predation hypothesis is that larval fish are minor constituents of the zooplankton community and that a great many zooplankton and planktivorous fishes eat larval fish. Moreover, eggs and yolk-sac larvae that have internal food supplies and do not die from starvation sustain mortality rates which are greater than first-feeding larvae, and this mortality can be due only to predation (Hunter 1984). Egg and larval predators have been identified in the field, and losses due to predation by copepods (Lillelund and Lasker 1971; Brewer et al. 1984), euphausids (Theilacker and Lasker 1974; Brewer et al. 1984), amphipods (Brewer et al. 1984); jellyfish (Möller 1984; Van der Veer 1985; Purcell et al. 1987), cnidarians and ctenophores (Purcell 1985), and fish (Frank and Leggett 1984; Alheit 1987) have been assessed. Planktivorous fish have been considered to be the cause of as much as 50% of the observed mortality of eggs of the Peruvian anchoveta, *Engraulis ringens* (Alheit 1987).

The objectives of this study were to (1) examine larval mortality rates of the Pacific sardine, *Sardinops sagax caerulea*, population, (2) compare these rates with the mortality rates of northern anchovy during the same period, and (3) investigate interannual variation of larval sardine mortality rates. Three possible hypotheses to explain annual variation in larval sardine

mortality rates were investigated. These were the stable ocean hypothesis (Lasker 1978, 1981) which proposes that periods of calm weather are necessary to allow first-feeding larvae to find aggregated food patches, the transport hypothesis (Hjort 1913) which proposes that currents may sweep larvae out of suitable feeding areas, and the predation hypothesis (Hunter 1984) which proposes that the abundance of predators on eggs and larvae will affect mortality rates.

These hypotheses were further tested to determine whether any factor can be used to predict recruitment. The potential predators of eggs and larvae that I considered were adult northern anchovy, Pacific sardine, and Pacific mackerel, *Scomber japonicus*. Jack mackerel were excluded as a potential predator because of their offshore distribution.

Methods

Larval Mortality

I estimated sardine larval mortality rates from the decrease in numbers of larvae in successive size intervals found in 1-m, 500-μm-mesh oblique ring net tows collected by the California Cooperative Oceanic Fisheries Investigation (CalCOFI). Size–frequency distributions of larvae from 3.9 to 16.2 mm were converted to age–frequency distributions using growth rates determined in the laboratory for yolk-sac larvae and in the field for feeding larvae.

Oblique net tows were taken during a series of monthly cruises beginning in 1951 and ending in 1966. Quarterly CalCOFI cruises were initiated in 1961. After 1967 the numbers of sardine larvae collected were insufficient for estimating mortality rates.

Abundance in each length class was estimated using a weighted negative binomial model (Bissell 1972; Zweifel and Smith 1981; Lo 1985). I adjusted the numbers of larvae in ichthyoplankton net collections for the following possible biases: extrusion through the mesh (Lenarz 1972, 1973; Lo 1983), day–night difference in avoidance (Lo 1985), and volume of water filtered at each depth (Kramer et al. 1972).

Duration of yolk-sac stage was determined from temperature-dependent growth rates calculated using the model of Zeifel and Lasker (1976). Sardine larvae hatch 2.24 d after fertilization at 17°C and reach the first-feeding stage in 5 d at a larval length of 6.2 mm (Zweifel and Lasker 1976). As we know from daily increments in their otoliths, sardine larvae reach a length of 15 mm (live standard length) in about 23 d after hatching (Butler and de Mendiola 1985; Castillo et al. 1986; Butler 1987).

Because estimates of the ages of fish larvae stored in formalin for more than 20 yr are unattainable from otoliths, I estimated ages by the duration of each length interval from growth rates back-calculated from increment widths in the otoliths of juveniles hatched in each month of the year (Butler 1987). The daily formation of these increments was demonstrated by Butler and de Mendiola (1985) and Butler (1987). I used a Laird–Gompertz curve to describe the growth rates of larval sardine:

$$(1)\quad L_t = L_\infty\,[L_{ff}/L_\infty]\; e^{-a_m\cdot(t\,-\,t_{ff})}$$

where L_t is the live length at age t days from first feeding, L_∞ is the asymptotic length at metamorphosis = 35 mm, L_{ff} is the length at first feeding and first increment formation = 6.2 mm, a_m is the seasonally dependent growth coefficient, and t is the time from first feeding in days or the number of daily increments.

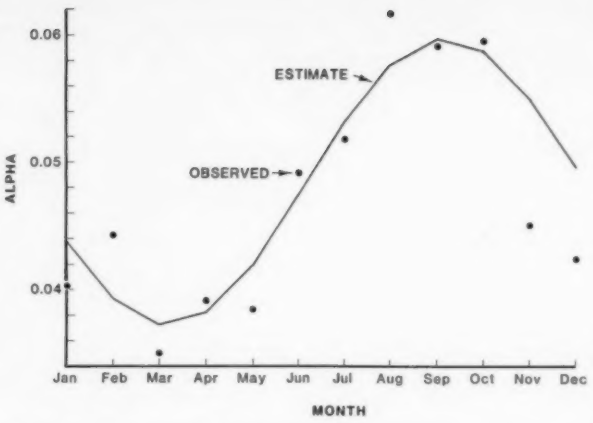

FIG. 1. Seasonal variation in larval growth parameter of the Pacific sardine. Smooth curve is sine function fitted to the data. See text for equation.

I used a sine curve to describe the seasonal growth coefficient (L_m) of the Gompertz curve (Laird 1967):

$$(2)\quad a_m = 0.0485 + 0.00113\cdot\sin(\,(\text{month} - 6.182)\cdot 0.5234).$$

I fitted equation (2) nonlinear regression, weighting the value of alpha of each month by the number of observations for that month (Fig. 1).

Survivorship of sardine larvae increases with age. Survivorship (S) at a given time (t) can be modeled using a Pareto hazard function:

$$(3)\quad S(t) = (t/u)^{-\beta}$$

where u is the time of hatching (Lo 1985). The observed mean daily production of larvae y_i is related to survivorship by

$$(4)\quad y_i = y_u(t/u)^{-\beta}.$$

β was estimated by taking the logarithm of both sides of the equation:

$$(5)\quad \ln(y_i) = B - \beta \ln(t_i/u).$$

Unlike that for anchovy, sardine mortality begins to decline at hatching. I used eight size intervals from hatching to a live size of about 15 mm to estimate β. I compared annual estimates of β for sardine larvae with the annual β for anchovy larvae estimated by Lo (1985) for the same geographic area.

Stable Ocean Hypothesis

To test the stable ocean hypothesis (Lasker 1975, 1981) that larval mortality rates are influenced by stability of the water column, I followed the procedure of Peterman and Bradford (1987), which is to correlate mortality rates with an index of water column stability.

To choose an appropriate stability index, I estimated a geographic center of sardine spawning from the position of positive plankton tows weighted by the number of sardine larvae in each tow. From 1951 to 1967 the latitude of the center of spawning varied from 25°56.4′N in 1965 to 32°11.4′N in 1959, with the overall mean at 28°24.5′N off northern Baja California (Table 1). Since wind data were not available for 29°N, stability indices were calculated from daily average wind speed at

TABLE 1. Geographic distribution of Pacific sardine larvae during 1951–67. Center of distribution is mean position of positive tows weighted by the number of larvae. Southern and northern positions are latitudinal limits of distribution.

	Center		Southern		Northern	
Year	Latitude North	Longitude West	Latitude North	Longitude West	Latitude North	Longitude West
1951	27°52.0′	115°24.7′	22°47.5′	110°22.0′	36°39.0′	122°26.0′
1952	27°59.6′	115°11.1′	24°19.0′	111°48.0′	35°57.0′	119°11.0′
1953	28° 4.3′	115°12.5′	25°33.3′	112°15.5′	34° 9.5′	119°31.8′
1954	29° 8.6′	116° 0.4′	24°21.3′	111°49.0′	35°54.3′	122°22.7′
1955	29°35.6′	116°40.3′	22°35.0′	110°15.1′	34° 8.5′	121° 9.0′
1956	29°10.1′	116° 1.9′	23°55.5′	111° 5.0′	33°47.7′	118°41.0′
1957	29°47.1′	115°55.0′	25°33.5′	112°18.0′	36°39.0′	122°26.0′
1958	31°24.8′	117° 2.4′	24°11.0′	112° 3.0′	36° 9.0′	121°50.0′
1959	32°11.4′	117°50.7′	23°56.0′	111° 4.2′	37°21.0′	124°22.0′
1960	31°10.4′	117°32.4′	24°19.0′	111°48.0′	34°15.2′	119°58.3′
1961	29°31.8′	115°40.6′	25°33.4′	112°18.5′	35°31.7′	121°25.2′
1962	28°27.8′	115°10.9′	26° 4.5′	112°48.0′	36°16.0′	121°40.0′
1963	29°26.1′	115°19.6′	25°38.0′	112°11.2′	36°10.7′	121°45.6′
1964	27°27.9′	113°36.2′	24° 2.0′	113°41.5′	35°38.2′	121°14.2′
1965	25°56.4′	112°27.2′	24°47.4′	112°20.3′	36°54.4′	121°52.9′
1966	27°33.6′	113°31.1′	25°33.6′	112°18.7′	36°10.4′	121°45.9′
1967	29° 0.1′	114°54.0′	25°34.0′	112°19.0′	31°42.1′	116°43.4′

30°N and 116°W. This wind speed index was determined from the number of consecutive 4-d periods of calm weather per month, derived from marine observations from 1953 to 1967.

Based on the analysis of Simpson and Dickey (1981), I used a wind speed of 10 m/s as a cutoff between calm weather and turbulent mixing. Wind speeds greater than 10 m/s produce turbulent mixing in the upper mixed layer which, according to Lasker (1975), should disrupt patches of microzooplankton necessary for successful first feeding of anchovy larvae. The stability index for each month was weighted by the monthly proportion of the annual production of sardine larvae. Missing values of larval production (35 of 204 months) were estimated from adjacent values.

Transport Hypothesis

In the California Current, transport has two components: a southerly component and a seasonal offshore component. To investigate Hjort's hypothesis that transport away from nursery grounds during the larval phase contributes to mortality of larvae, I calculated two indices of transport. Sea level has been shown to be an index of changes in the southerly transport of the California Current (Chelton 1980; Chelton et al. 1982), and offshore Ekman transport is an index of offshore transport (Parrish et al. 1981; Bakun 1985). Each index for each month was weighted by the monthly proportion of the annual production of sardine larvae.

Although no sea level index is available for the center of sardine spawning, the anomalies at one locality are strongly correlated with anomalies at other localities along the coast (Chelton 1980). Thus, the index calculated for San Diego may be considered representative of northern Baja California.

To investigate whether interannual variability in offshore transport influenced the observed increase in larval sardine mortality rates, I calculated an annual index of upwelling at 31°N, 119°W from monthly upwelling indices (Bakun 1973), weighted by the monthly proportion of the annual production of larvae.

Predation Hypothesis

To investigate the hypothesis that the total abundance of coastal pelagic planktivorous fishes affects larval sardine mortality rates, I regressed β on estimates of the total biomass of Pacific sardine by MacCall (1979), Pacific mackerel by Parrish and MacCall (1978), and northern anchovy by Methot (1986). I also regressed β on the biomass estimates of each species. Estimates of total sardine biomass (Table 2) and biomass of recruits at age 2 are from virtual population analysis estimates derived from landings and are independent of the number of sardine eggs and larvae in plankton samples.

The abundance of alternative prey has been proposed to affect the strength of predation by pelagic fish on larval fish (Pepin 1987). To test this hypothesis, I calculated a predator to prey ratio from the total biomass of pelagic fish and zooplankton volumes (Table 3).

To investigate the hypothesis that the abundance of predators affects recruitment through predation on young-of-the-year sardines, I regressed an index of recruitment against the total biomass of coastal pelagic fishes (as defined above) with a 2-yr time lag. The index that I used was the logarithm of the ratio of sardine recruits at age 2 to spawning biomass. Thus, the abundance of predators during the year of spawning was compared with the strength of recruitment 2 yr later. This index was also compared with the biomass of each species to detect any interspecific affects.

Correlation Analyses

Since correlation analysis cannot reveal cause and effect, any correlation may be a result of a correlation with another variable. To test for alternative hypotheses, I performed a stepwise multiple correlation of β and recruitment success on several factors. These factors included temperatures at 10 m (Table 4), zooplankton volumes, latitude of center of spawning, Scripps Pier salinities, sea level anomalies, wind speed indices, upwelling indices, anchovy biomass, Pacific sardine biomass, and Pacific mackerel biomass. Small zooplankton volumes, rou-

TABLE 2. Time series of estimated larval mortality coefficients and total biomass estimates (metric tons, t) of Pacific sardine and northern anchovy during 1951–67.

	Pacific Sardine			Northern anchovy			
Year	Biomass (10^3 t)[a]	Larval β	SE	Biomass (10^3 t)[b]	Larval β	SE	Total biomass (10^3 t)[c]
1951	277	1.74	0.18		0.85	0.15	277
1952	136	2.02	0.25		0.88	0.20	136
1953	202	1.54	0.34		0.95	0.10	202
1954	239	1.77	0.30	45	1.16	0.24	284
1955	170	2.66	0.18	32	1.01	0.17	202
1956	108	2.23	0.33	24	0.88	0.20	131
1957	90	1.65	0.40	88	0.94	0.10	178
1958	177	2.53	0.18	146	0.88	0.08	323
1959	122	2.69	0.18	111	1.18	0.06	233
1960	88	2.35	0.21	66	1.80	0.23	154
1961	54	2.71	0.42	130	1.55	0.16	184
1962	27	2.77	0.36	194	1.08	0.23	221
1963	21	3.79	0.23	204	0.81	0.04	225
1964	11	3.07	0.21	235	1.44	0.21	246
1965	3	3.54	0.28	257	2.42	0.12	260
1966		3.63	0.15	226	1.84	0.04	226
1967		3.77	0.23	195	2.15	0.08	195

[a]From MacCall (1979).
[b]From Methot (1986).
[c]From Lo (1985).

TABLE 3. Annual weighted mean volume of small zooplankton ($cm^3/1000\ m^3$) in 1-m net tows positive for sardine larvae. Mean value weighted by number of sardine larvae collected in tow.

Year	Mean	SD	Low	High
1951	113	63	13	609
1952	154	125	17	1144
1953	168	112	15	1058
1954	145	122	16	1660
1955	209	354	17	6509
1956	289	240	17	1875
1957	165	110	13	2298
1958	110	71	9	532
1959	100	103	7	646
1960	227	164	10	1814
1961	157	93	6	652
1962	164	174	17	1071
1963	382	270	17	1231
1964	225	289	17	1547
1965	372	132	14	977
1966	211	73	17	926
1967	367	272	5	2215

TABLE 4. Annual weighted mean temperature of stations positive for Pacific sardine larvae during 1951–67. Temperature at each station weighted by the number of larvae collected at that station.

	Temperature (°C)					
Year	Mean	Low	High	SD	Stations	Larvae
1951	16.8	11.3	26.4	2.5	162	6059
1952	17.1	11.2	24.4	1.9	203	5512
1953	16.7	12.1	25.2	2.1	175	3414
1954	16.4	12.9	23.7	2.0	355	7108
1955	15.6	12.8	20.2	1.2	255	4358
1956	17.5	11.9	27.4	3.5	169	2692
1957	19.1	13.0	24.8	2.8	173	2712
1958	19.0	11.9	27.3	2.5	193	3824
1959	18.7	12.7	25.0	3.2	170	2079
1960	19.4	10.7	25.0	2.4	138	3723
1961	17.3	12.6	22.0	1.7	53	761
1962	17.7	12.8	23.0	2.2	57	1006
1963	19.7	10.9	24.6	3.2	99	2141
1964	19.6	12.2	25.2	2.9	86	4892
1965	23.2	11.3	26.5	2.0	105	12272
1966	22.4	11.8	24.7	3.2	139	4900
1967	15.7	13.0	20.7	1.8	24	684

tinely measured in the CalCOFI program, are the displacement volumes of the zooplankton taken in ichthyoplankton tows after large gelatinous organisms, such as medusa and salps with individual volumes exceeding 5 mL, are removed (Kramer et al. 1972). These volumes are assumed to be a measure of potential predators rather than larval food which must be collected with 75-μm-mesh nets (Owen et al. 1990).

Temperature, zooplankton volumes, and the geographic positions were weighted by the number of sardine larvae at each station. Weighting produces values representative of the environment experienced by most sardine larvae, but it is not confounded with mortality, which is calculated from abundance of larvae in length classes without regard to their geographic position.

The testing of the significance of correlation coefficients assumes that each observation in the data set is independent. For time series, this requires that the autocorrelation coefficients be small for lag periods greater than zero. This is not the case for time series of populations with generation times greater than the periodicity of sampling. Thus, the time series of anchovy biomass and β are both autocorrelated at a time lag of 1 yr ($R = 0.793$ for anchovy and $R = 0.631$ for β). For autocorrelated time series, Bayley and Hammersley (1946) have proposed a corrected number of independent observations, N^*:

$$(6)\quad 1/N^* = 1/N + 1/N^2 \sum_{j=1}^{N-1} (N-j)\,[P_j\tau]^2$$

where N is the total number of observations and $(P_j\pi)$ is the autocorrelation coefficient of the j^{th} lag of period. Blackman and Tukey (1958) suggested that j not exceed 1/10 N because of the small number of observations included in the autocorrelations as j approaches $(N-1)$. The significance levels that I use are based on Bayley and Hammersley's (1946) procedure as modified by Blackman and Tukey (1958) (see also Sutcliffe et al. 1976).

Results

Larval Mortality

The log transform for abundance as a function of age is best fit with a straight line (Fig. 2). Thus, the probability of survival of sardine larvae increases with age. The age-specific mortality coefficient, β, varied during 1951–67 (Table 2). The mean value for the 17-yr time series was 2.61 and the variance was 0.562.

Mortality rates of sardine larvae were significantly greater than those of anchovy larvae in the same area in 14 of 17 yr during 1951–67 (Fig. 3). Survival from yolk-sac larvae to 20-mm larvae ranged from 0.05 to 3.1% during 1951–67. During the same period, survival of anchovy larvae from first feeding to 19 mm length ranged from 0.2 to 10.6% (Lo 1985).

Stable Ocean Hypothesis

The estimated mortality rates of sardine larvae showed no relationship to the stability of the water column during 1951–66. There was no significant correlation ($R^2 < 0.03$, $N^* = 14$, $P > 0.37$) with the weighted index of calm weather and the larval sardine mortality coefficient, β.

Salinities of seawater have been correlated with the catch of 3-yr-old Pacific sardine (Walford 1946). This correlation was over a limited time series and attempted to relate the apparent year-class size of Pacific sardine to salinity as an index of upwelling, and hence food availability. Neither larval sardine mortality coefficient, β, calculated here, nor sardine recruitment was correlated with either annual mean Scripps Pier salinities or the annual anomaly from 1951 through 1967 ($R^2 = 0$, $N^* = 14$, $P > 0.10$). Thus, no support was found for the hypothesis that salinity as an index of upwelling affects larval mortality or predicts recruitment.

Transport Hypothesis

Advection of fish larvae away from coastal nursery areas may lead to higher mortality. However, neither the mortality rate of sardine larvae ($R^2 = 0.20$, $N^* = 15$, $P = 0.09$) nor the recruitment of 2-yr-olds ($R^2 = 0.08$, $N^* = 12$, $P = 0.37$) was correlated with the upwelling index at 31°N. Thus, this time series does not support the hypothesis that offshore transport is a determining factor of mortality rates in Pacific sardine larvae.

The mortality rate of sardine larvae showed no correlation ($R^2 = 0.03$, $N^* = 14$, $P > 0.10$) with the sea level anomaly as an index of transport. However, the recruitment of 2-yr-olds showed a positive correlation ($R^2 = 34.3$, $N^* = 13$, $P < 0.05$) with the average sea level anomaly during 1951–67. If the average sea level anomaly is compared with recruitment using a 2-yr time lag, to test the effect of southerly transport during the year that the eggs were spawned, then the correlation is negative, but not significant ($R^2 = 0.074$, $N^* = 13$, $P > 0.10$). Evidence for the hypothesis that southerly transport determines sardine recruitment will be discussed below.

Predation Hypothesis

A corollary of the predation hypothesis is that mortality rates of fish larvae should increase when predator abundance increases. Important predators of planktonic larvae are other zooplankton and planktivorous fish. The mortality of sardine larvae increased in years of high zooplankton volumes (Table 3). Small zooplankton biomass was associated with 36% of the interannual variability of sardine larval β ($N^* = 12$, $P < 0.05$). This indicates a positive relationship between mortality rates and the abundance of zooplankton predators.

Mortality rates of sardine larvae increased over the period 1951–67 (Fig. 3). The biomass of northern anchovy also increased during this period (Table 2). Mortality rates of sardine larvae were positively correlated with anchovy abundance (Fig. 4). The increase in biomass of northern anchovy accounted for 58% of the interannual variability of larval sardine mortality rates ($N^* = 6$, $P < 0.01$).

The abundance of alternative prey affects the strength of fish predation on larval fish. The mortality rate of sardine larvae was positively correlated with the predator prey ratio (pelagic fish biomass/zooplankton volumes) ($R^2 = 0.47$, $N^* = 12$, $P < 0.01$).

Total pelagic fish biomass was significantly negatively correlated with the index of sardine recruit-per-spawn lagged by 2 yr ($R^2 = 0.39$, $N^* = 12$, $P = 0.03$). This correlation is not simply due to a similar time trend in both variables. Pelagic fish biomass varied with a cyclic pattern from 1951 to 1962 (Fig. 5). This time series was significantly negatively autocorrelated at a time lag of 2 yr. The recruitment index of Pacific sardine varied in a similar, but opposite, cycle from 1951 to about 1958. Afterwards, recruitment remained low through 1962.

Biotic factors, rather than abiotic factors, appear to affect larval sardine mortality. The relationship between anchovy biomass and zooplankton biomass seems to explain the observed interannual variability in sardine mortality rates. Larval sardine mortality rates were significantly negatively correlated with both anchovy biomass ($R^2 = 0.63$, $N^* = 8$, $P = 0.001$) and zooplankton biomass ($R^2 = 0.40$, $N^* = 14$, $P = 0.01$). However, larval sardine mortality rates were not correlated with either the total Pacific mackerel biomass ($R^2 = 0.01$, $N^* = 13$, $P > 0.10$) or with the biomass of Pacific mackerel recruits (data from Parrish and MacCall 1978).

The dominant period of the oscillations in pelagic fish biomass (Fig. 5) is 2 yr. This is about the life span of the northern anchovy. To investigate whether northern anchovy affects sardine recruitment, I performed a stepwise multiple correlation analysis on total pelagic fish and anchovy biomass. In this analysis, anchovy biomass was more strongly correlated with sardine recruitment than total pelagic fish biomass ($R^2 = 0.37$, $N^* = 6$, $P = 0.08$). Because of the significant correlation between total pelagic fish biomass and anchovy biomass, it is difficult to separate the two hypotheses that predation by northern anchovy or predation by all pelagic fish on late larvae and juvenile sardines determines recruitment. However, the predation hypothesis is supported by this analysis.

Alternative Hypotheses

The correlations found in the preceding sections may not be due to cause and effect but rather to correlations with some

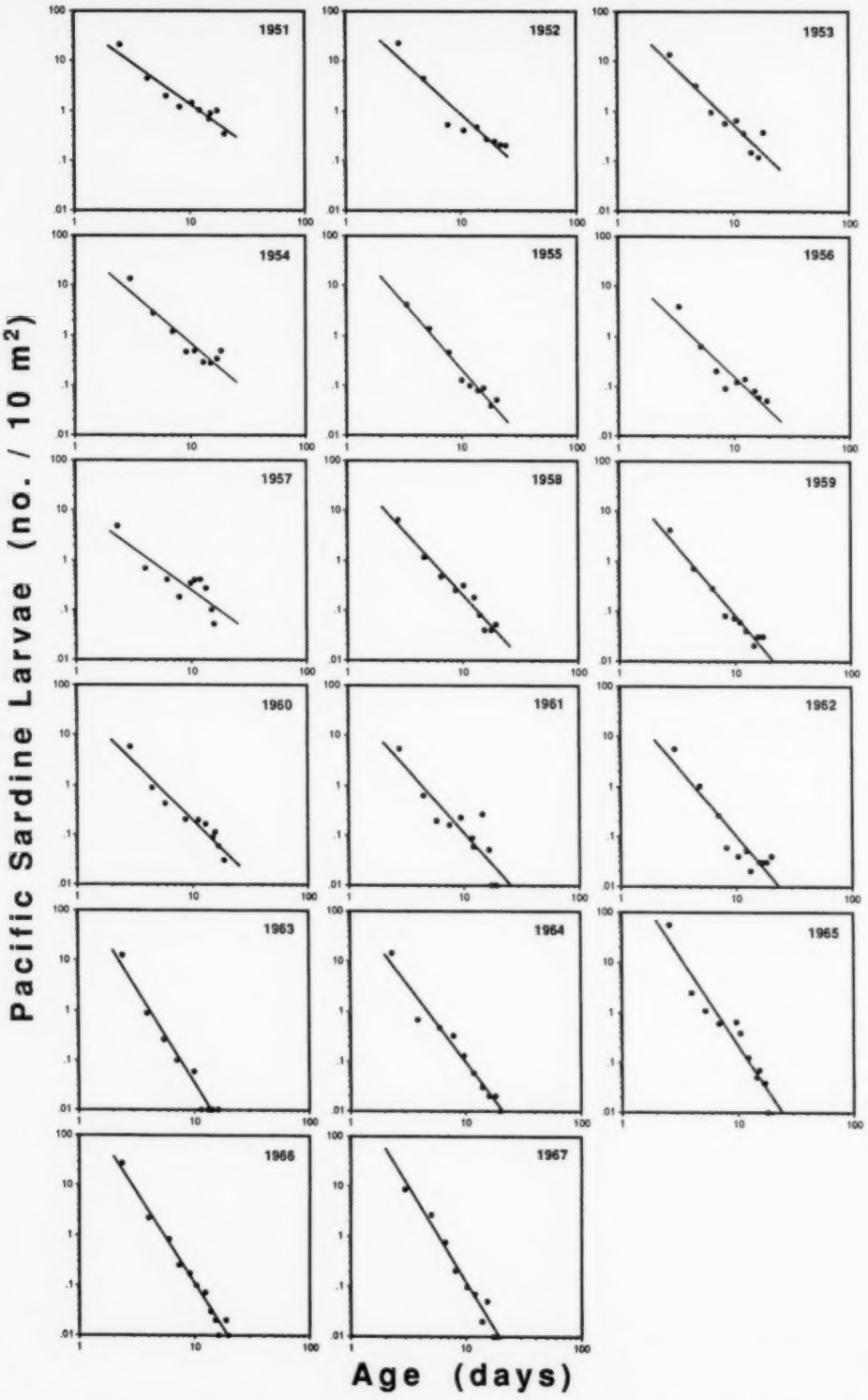

FIG. 2. Abundance of Pacific sardine larvae as a function of age for 1951–67.

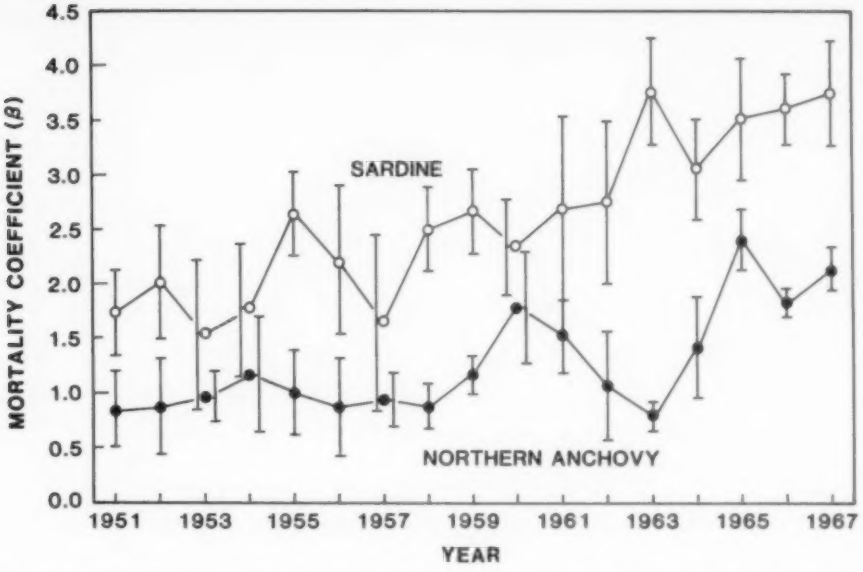

FIG. 3. Increase in the larval mortality coefficient, β, during 1951–67 for Pacific sardine and northern anchovy.

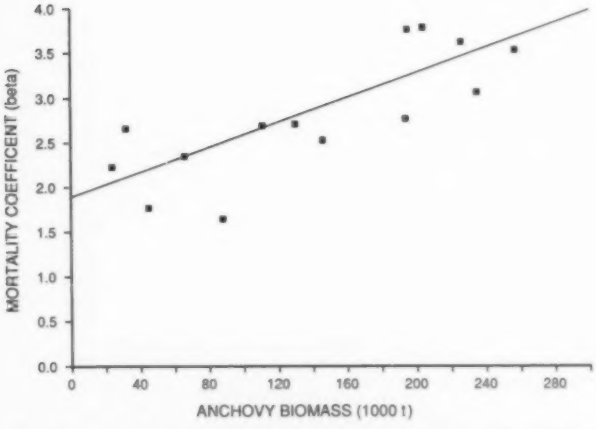

FIG. 4. Correlation of interannual variation of the larval mortality coefficient, β, of Pacific sardine and total biomass of northern anchovy.

other factor which affects mortality and recruitment. To test for an alternative explanation, I performed correlation analyses with some of the other factors which might be operating. These were temperature, geographic locality, small plankton abundance, salinity, turbulent mixing, and offshore transport.

Because larval sardine growth rates are correlated with temperature (Castillo et al. 1986) and growth rates determine the duration of larvae in each length class, temperature may affect mortality in an important way. Temperature is also used to calculate embryonic growth rates, which are then used to calculate mortality rates ($dN/dt = dN/dL \cdot dL/dt$ where dL is a length interval); a positive correlation with mortality rates is expected if temperature is confounded with mortality rates. However, lower temperature may slow the rate of development and extend the time that larvae experience high mortality at small sizes. Thus, a negative relationship between mortality and temperature is also conceivable. At stations yielding sardine larvae, temperatures ranged from 10.7 to 27.4°C. The annual weighted means ranged only from 15.6 to 23.2°C (Table 4). These temperatures were not significantly correlated with larval sardine mortality rate ($R^2 = 0.172$, $N^* = 15$, $P > 0.10$). Thus, I reject temperature as an important factor contributing to larval mortality.

To investigate whether there were significant interactions between mortality rates and any other variable, I tested the relationship using stepwise multiple correlation with temperature, latitude, small plankton volume, and Scripps Pier salinity anomalies, an index of turbulent mixing derived from wind data, an index of offshore transport, and the biomass of sardine, the biomass of anchovy, and the biomass of Pacific mackerel. The only significant correlation detected was between larval sardine mortality rates and anchovy biomass, and this correlation was positive. The coefficient of determination of β and anchovy biomass was 0.63 ($N^* = 9$, $P = 0.001$). The multiple correlation of β and anchovy biomass and small zooplankton volumes as a second correlate was not significant ($R^2 = 0.74$, $N^* = 9$, $P = 0.07$). Thus, the abundance of anchovy is the most important variable tested that explains the interannual variability in larval sardine mortality rates.

Larval mortality rates are only one factor in the process of recruitment. Because sardines enter the fishery at age 2, considerable mortality must occur between the larval phase and recruitment. To test whether larval mortality rates are a predictive indicator of recruitment, I regressed them against biomass at age. The association was not significant, although the sign of the relationship was negative as expected.

To investigate whether recruitment declined due to density-dependent effects on larval mortality rates (β), I regressed the logarithm of ratio of recruits at age 2 and the spawning biomass lagged by 2 yr as an index of recruitment success on both sar-

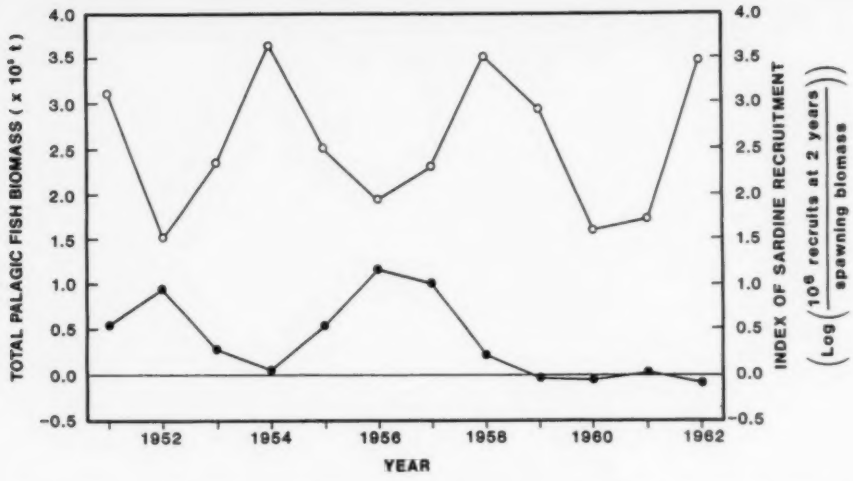

FIG. 5. Time series of total pelagic fish biomass (open circles) (Pacific sardine, northern anchovy, and Pacific mackerel) and recruitment index (solid circles) of Pacific sardine.

dine spawning biomass and β. Neither sardine biomass ($N^* = 11$, $P = 0.717$), nor β ($N^* = 10$, $P = 0.16$), nor the two variables in combination predicts recruitment success.

I also tested whether any other environmental factor was a reliable predictor of recruitment success: temperature, zooplankton volumes, latitude of center of spawning, sardine biomass, anchovy biomass, Pacific mackerel biomass, total pelagic fish biomass (anchovy, sardine, and Pacific mackerel), average sea level anomaly, and wind speed index. Again, total pelagic fish biomass ($N^* = 12$, $P = 0.03$) and anchovy biomass ($N^* = 9$, $P = 0.08$) were significantly negatively correlated with sardine recruitment success. Thus, no environmental factor, other than the abundance of predators, predicts sardine recruitment success.

Discussion

Larval Mortality

The mortality of Pacific sardine larvae was greater than that of northern anchovy larvae from 1951 to 1967 ($T = 9.09$, df = 16, $P << 0.01$). Others have reported similar results. Smith (1973, 1985) found that the ratio of the abundance of Pacific sardine larvae to sardine eggs in ichthyoplankton net tows was less than the corresponding ratio in northern anchovy, indicating that the mortality rates of larval sardines are indeed higher than those of larval anchovy. Smith et al. (1989) found higher mortality in sardine eggs than in Peruvian anchovy eggs in the same water mass.

Analysis of swimming energetics (Webb and Weihs 1986) and distribution of prey items (Vlymen 1977) also suggest greater mortality rates for faster growing larvae. Faster growing larvae must search greater volumes of water to obtain more food, and in so doing are at greater risk to predation than slower growers. On the other hand, lesser mortality rates for faster growing fish larvae are suggested by the relationship of particle size and mortality (Brooks 1968; Peterson and Wroblewski 1984; Kirkpatrick 1984), i.e. mortality in the sea is inversely related to body size. Faster growing fish larvae are subject to high mortality rates associated with small size but for a shorter period of time than slow-growing larvae. Because the mortality of the faster growing sardine larvae is greater than the mortality of the slower growing anchovy larvae, the energetic arguments of Webb and Weihs (1986) and Vlymen (1977) are supported by this analysis.

Behavioral differences observed in the laboratory may explain the observed differences in mortality rates. At a given age, fewer sardine larvae than anchovy larvae escape attack by adult anchovy (Butler and Pickett 1987). If predation contributes a significant fraction of larval mortality, then the greater vulnerability to visual predators of sardine larvae would contribute to a greater mortality rate. The observation by Brownell (1985) that Cape anchovy, *Engraulis capensis*, cannibalize larvae and juveniles as large as 50% of their own length supports the idea that anchovy may contribute to sardine mortality at lengths longer than the 20 mm considered here.

Stable Ocean Hypothesis

The lack of correlation between the number of calm days at 27°N, 116°W and larval sardine mortality rates does not support the hypothesis that periods of calm weather are necessary for the survival of all larval fish. This lack of correlation may be due in part to the absence of a strong signal in wind speed at this location. In the California Current regions, most of the strong wind events are related to storms which originate in the North Pacific. These storms reach southern California only infrequently, and their effects are less in northern Baja California.

In order for mixing to have an impact on larval mortality rates or recruitment, it must affect a significant portion of the population. Because most sardine spawning was well to the south of Point Conception, it is unlikely that storms and the resulting instability of the water column play a major role affecting the survival of sardine larvae in the California Current. Thus, Peterman and Bradford's (1987) conclusion that Lasker's stable ocean hypothesis accounts for a significant fraction of the interannual variability in the mortality of anchovy larvae is not extended to sardine larvae by this analysis.

The stable ocean hypothesis may explain the mortality of first-feeding anchovy larvae, but it does not explain recruitment

(Peterman et al. 1988). Evidence that starvation is not an important factor after the first-feeding stage has been presented by Owen et al. (1990) who found no difference in starvation rates, growth rates, or mortality rates of anchovy larvae in two areas of the California Current characterized by contrasting biomasses of plankton as collected by 45-μm-mesh nets. Lack of variation of larval anchovy growth rates between El Niño and non-El Niño years also casts doubt on the starvation hypothesis (Butler 1989).

Transport Hypothesis

Hjort's (1913) hypothesis that year class strength was determined early in life of fishes and that the sources of mortality of larval fish were due to starvation of first-feeding larvae or to transport out of feeding grounds has been the basis of much fisheries research. Based on the analysis of larval mortality rates and transport both southerly and offshore, there is no support for either component as a mechanism determining sardine larval mortality rates. Nor is there support for the hypothesis that offshore transport affects recruitment success to the adult population.

Whether southerly transport affects recruitment is debatable. The sea level anomaly, as an index of transport, has been correlated with recruitment to the Pacific mackerel population (Sinclair et al. 1985). Recruitment of 2-yr-old sardines is also correlated with the sea level anomaly (MacCall 1979 and above). This correlation may be due to the increased availability of 2-yr-old sardines off Southern California during the El Niño years of 1957–58 (MacCall 1979). If the El Niño years are excluded from the analysis, there is no correlation. However, eliminating El Niño years might exclude the conditions which would produce the hypothesized effect.

Examination of the aged landings (MacCall 1979, table 1) suggests that either the 1957 and 1958 year-classes were overrepresented or those year-classes incurred greater than normal mortality in the ensuing years. Furthermore, if the sea level anomaly is compared with recruitment, using a 2-yr time lag to test the effect of southerly transport during the year that the eggs were spawned, then the correlation is negative, but not significant. Thus, Hjort's (1913) second hypothesis that transport away from nursery grounds is not strongly supported by this analysis.

Predation Hypothesis

Although the predation hypothesis is supported by the evidence presented here, none of the other hypotheses can be categorically rejected. However, no correlation other than with predators could explain the observed trend in larval mortality rates. Predation is also consistent with the observed trend in recruitment.

Cannibalism of eggs and larvae in anchovies is well known (de Ciechomski 1967; Hayasi 1967; Loukashkin 1970; Hunter and Kimbrell 1980; MacCall 1980; Santander et al. 1983; Alheit 1987; Smith et al. 1989). In the northern anchovy, cannibalism can account for 20–28% of the observed egg mortality (Hunter and Kimbrell 1980). In an analysis of cannibalism by the Peruvian anchoveta, Alheit (1987) found that cannibalism accounted for 22% of the egg mortality. Stomachs of Pacific sardine in the same area also contained anchoveta eggs. On a per-weight basis, Alheit (1987) estimated that sardines may consume as many as or more anchoveta eggs than the anchoveta. Together, the two species may account for about 50% of the daily anchoveta egg mortality.

If northern anchovy filter their own eggs, then they must certainly also feed on other fish eggs and larvae. Perhaps because of the thin integument of larval fish and because of rapid digestion rates, fish larvae are rarely found in the stomachs of anchovy (Hunter and Kimbrell 1980). Fish eggs reported in the stomachs of anchovy by Loukashkin (1970) were not identified. Northern anchovy occurs with sardine during spawning (Ahlstrom 1967) and thus could consume significant numbers of eggs and larvae. Cannibalism is also consistent with the observed trend in anchovy larval mortality rates, but this effect was discounted by Peterman and Bradford (1987) due to the confounding of the slope (mortality) and the intercept (spawning biomass) of larval production and because mortality was more strongly correlated with water column stability. However, Pepin (1987) extended the analysis to predator to prey ratios and found that anchovy larval mortality rates were related to predation by total pelagic fish biomass.

Smith (1985) listed three situations in which cannibalism by schooling pelagic fishes would be effective in population control: (1) directed filtering behavior on dense aggregations of eggs, (2) encounter with other schools' patches of eggs, and (3) anchovy population switching from biting to filtering behavior. The correlation of mortality rates with predator to prey ratios is support for the later scenario. The same three situations also apply to predation of schooling pelagic fish on the eggs and larvae of other fishes, and the latter two scenarios are consistent with the observed trends in Pacific sardine larval mortality and northern anchovy biomass.

Conclusions

Competition between adults (Murphy 1966) or between larvae (Marr 1960) has been suggested as the important interactions between sardine and anchovy. Between 1951 and 1967, it is unlikely that the abundance of either species or their total biomass could have affected their food supply significantly. The biomass of sardines is far below its maximum in the 1930's. It is also unlikely that the biomass of fish larvae ever affects larval food densities (Cushing 1983). But Jones (1989) argued that larval fish may consume a significant fraction of secondary production and that resource limitation is possible for larval fish. Owen et al. (1990) found no difference in larval growth rates between sites with contrasting microzooplankton densities. The correlation of larval sardine mortality with anchovy abundance indicates an important interaction between the two species which has not been documented previously.

The decline of the Pacific sardine fishery during the late 1940's and 1950's has been ascribed to overfishing during periods of poor recruitment (Clark, in Clark and Marr 1955; Murphy 1966; MacCall 1979). Marr (1960) found no obvious relationship between parent stock size and recruitment and concluded that environmental change was responsible for recruitment failure and the decline of the Pacific sardine stock. Iles (1973) also suggested climatic change as the agency of recruitment failure of the Pacific sardine.

Analyzing the waning years of the sardine fishery, MacCall (1979) noted a "string of poor recruitments since 1958. . .remarkable in its duration and magnitude." The increase in larval mortality rates during the time period is also remarkable for its duration and magnitude. The correlation of the larval mortality rates with anchovy biomass indirectly sug-

gests that the two species interact by predation. Anchovy predation on eggs and young larvae (<20 d) would produce the observed correlation between anchovy biomass and larval sardine mortality rates. Predation on older larvae (>20 d) and young juveniles would explain the negative correlation between sardine recruitment and the combined biomass of pelagic fishes and would largely explain the string of poor recruitments observed in the 1960's.

Of the three hypotheses advanced to predict larval mortality rates, water column stability and starvation of larvae, transport away from nursery grounds, and predation, only predation explains the observed trends in larval sardine mortality rates and recruitment success.

Acknowledgments

Andrew Bakun, Pacific Fisheries Environmental Group, provided wind speed data. Rich Methot provided estimates of the northern anchovy biomass. I especially thank Nancy Lo for her assistance and both Paul Smith and John Hunter for their advice. Waldo Wakefield, Gail Theilacker, Al Myrick, and William Bayliff (Inter-American Tropical Tuna Commission) read and commented on the manuscript.

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Discrimination among Atlantic Coast Populations of American Shad (*Alosa sapidissima*) Using Mitochondrial DNA

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Nolan, K., J. Grossfield, and I. Wirgin. 1991. Discrimination among Atlantic coast populations of American shad (*Alosa sapidissima*) using mitochondrial DNA. Can. J. Fish. Aquat. Sci. 48: 1724–1734.

We used restriction endonuclease analysis of mitochondrial DNA (mtDNA) to differentiate among spawning stocks of American shad (*Alosa sapidissima*). Highly purified mtDNA was isolated from shad from four major spawning rivers: the St. John's (Florida), the Delaware, and the Hudson in the United States and the Miramichi in New Brunswick, Canada. Primarily four-and-five-base-cutting restriction enzymes were used to prepare both individual enzyme profiles and composite genotypes. Three separate spawning stocks, St. John's, Delaware–Hudson, and Miramichi, could be distinguished based on frequency differences in mtDNA genotypes generated by single restriction enzyme digests. We could not distinguish Delaware from Hudson River shad. Only a single definitive restriction site polymorphism was observed among all samples, but polyacrylamide gel electrophoretic mobility variants were common. *Eco* RI, *Dde* I, and *Rsa* I revealed stock-specific mtDNA genotypes. The frequencies of some genotypes occurred in latitudinal clines. Fifty-seven of 81 fish showed individual-specific composite genotypes. Geographic partitioning of genotypes suggests that mtDNA analysis may be useful for the identification of some American shad stocks and their relative contributions to mixed coastal fisheries.

Les auteurs ont utilisé des patrons des endonucléases de restriction de l'ADN mitochondrial (ADNmt) pour distinguer des stocks reproducteurs d'alose savoureuse (*Alosa sapidissima*). Ils ont isolé de l'ADNmt fortement purifié d'aloses, capturées dans quatre importants bassins de pointe: la St. Johns (Floride), le Delaware et l'Hudson (É.-U.) et la Miramichi (Nouveau-Brunswick, Canada). Ils ont premièrement utilisé des endonucléases de restriction hydrolyseurs à quatre et cinq bases pour préparer des zymogrammes individuels et des génotypes composés. Ils ont ainsi pu identifier trois différents stocks reproducteurs, soit ceux de la St. John's, du Delaware–Hudson et de la Miramichi, d'après les différences des fréquences dans les génotypes de l'ADNmt obtenus par hydrolyse simple à l'aide d'endonucléases de restriction. Ils n'ont toutefois pu distinguer les aloses du Delaware et de l'Hudson. Ils n'ont observé qu'un seul polymorphisme définitif de site de restriction parmi tous les échantillons, mais des variantes de mobilité électrophorétique sur gel de polyacrylamide étaient communes. *Eco* RI, *Dde* I et *Rsa* I ont révélé des génotypes de l'ADNmt stock-spécifiques. La fréquence de certains génotypes suivait des clines latitudinaux. De 81 poissons, 57 montraient des génotypes composés spécifiques à des individus. Le partitionnement géographique des génotypes porte à croire que l'analyse de l'ADNmt pourrait servir à l'identification de certains stocks d'alose savoureuse et leurs contributions relatives aux pêches côtières polyspécifiques.

Received October 5, 1989
Accepted March 13, 1991
(JA334)

Reçu le 5 octobre 1989
Accepté le 13 mars 1991

The American shad (*Alosa sapidissima*) is an anadromous clupeid fish of considerable economic importance throughout its western Atlantic range, which extends from the St. John's River in northern Florida to the St. Lawrence River in Canada (Walburg and Nichols 1967; Scott and Crossman 1973). Historically, spawning runs have been documented in at least 23 rivers within this range (Walburg and Nichols 1967). Shad spend their post-juvenile lives (4–6 yr) in the ocean before they return to their natal rivers in the spring to spawn (Talbot and Sykes 1958; Neves and Depres 1979). In the warmer months, adult fish are found in the Bay of Fundy, the St. Lawrence estuary, and off the Newfoundland and Labrador coasts. Mark–recapture studies of fish tagged during the summer in the Bay of Fundy have framed the overall pattern of shad migration and have provided recaptures from all the major shad spawning rivers along the Atlantic coast of North America including those in Florida and Georgia (Dadswell et al. 1987).

Commercial landings of shad have declined over the past 100 yr, probably reflecting a reduced population size. This decline has been particularly severe in the spawning rivers south of the Delaware Bay. While the overall catch for the Atlantic seaboard has decreased, stocks in the Connecticut, Hudson, and Delaware rivers appear to have stabilized (for a review of American shad catch statistics, see Cooper 1984). Asynchronous trends in catch data among these rivers probably reflect a history of differential recruitment success. Additionally, latitudinal variation in reproductive strategies have been demonstrated among shad from different spawning systems (Leggett and Carscadden 1978). Based on these observations, differential management of shad from the individual spawning

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systems might optimize utilization of this resource. Stock discrimination was identified as an area of highest priority by the workshop on critical data needs for shad research on the Atlantic coast of North America (Cooper 1984).

Multivariate analysis of meristic characters has revealed significant differences among spawning shad collected from the Hudson, Connecticut, Miramichi, and St. John (New Brunswick) rivers (Carscadden and Leggett 1975). Analysis of otoliths (Williams 1985) and multiple meristic and morphometric characters (Melvin 1984) have been used to assign the stock origin of shad collected in the mixed summer fishery in the Cumberland Basin, New Brunswick, to three broad geographic groups encompassing Canadian Atlantic, Mid-U.S. Atlantic, and South-U.S. Atlantic rivers. However, the identification of origin of fish by meristic analysis can be difficult due to the large overlap in individual counts, often negating the applicability of such analyses to the question of relative contribution to mixed fisheries. Although meristic and morphometric characters have a genetic component to their expression, they are also subject to temporal instability due to environmental influences (Blaxter 1984), thereby potentially requiring the annual recalibration of discriminators. For example, significant temporal variability in meristic counts among year classes of several species of alosids was reported in tributaries of the Chesapeake Bay (DesFosse and Loesch 1985), although Carscadden and Leggett (1975) found little difference in meristic counts among year classes of shad in the St. John River, New Brunswick.

Genetic approaches might eliminate the need for annual recalibration of discriminators. However, electrophoretic analysis of proteins did not reveal significant genetic heterogeneity among shad collected at several sites in the Chesapeake Bay (Sismour and Birdsong 1986) which is consistent with findings on other anadromous fish species, such as striped bass (*Morone saxatilis*) from this region (Sidell et al. 1980). Restriction analysis of mitochondrial DNA (mtDNA) affords advantages over protein electrophoresis for identifying fish stocks. Mitochondrial DNA evolves more rapidly than single copy nuclear genes (Brown et al. 1979), and thus, population-specific fixed mtDNA genotypes have been frequently observed in terrestrial animals (Avise et al. 1979) and freshwater fishes (Bermingham and Avise 1986). Mitochondrial DNA is transmitted maternally (Lansman et al. 1983), and in the absence of further mutation, all progeny of a single female share identical mtDNA genotypes. This permits easy assignment of maternal ancestry of individual fish although no information is generated concerning paternal lineage.

We tested whether a genetic mark, such as mtDNA genotypes, confirms the differentiation of the three regional shad spawning aggregations initially detected via morphological analysis. Mitochondrial DNA analysis of American shad populations by Bentzen and co-workers (1988, 1989) has suggested that overall levels of mtDNA sequence divergence among shad are low. However, they did detect significant differences in the distribution of several restriction site polymorphisms among shad. They also reported that some shad from southern rivers exhibited a larger mtDNA molecule than shad from northern rivers. Heteroplasmy was observed for a *Sal* I restriction site polymorphism and for a variant in the length of the mtDNA molecule. Our approach differed in that we used primarily four-and-five-base-cutting restriction enzymes, based on the assumption that they would produce a large number of small mtDNA fragments, and thus potentially reveal additional polymorphic markers.

Materials and Methods

Sample Collections and mtDNA Preparation

Samples were collected with hook and line from the St. John's River in Florida (38) and by drifting and anchored gill nets from the Delaware (29), Hudson (44), and Miramichi (15) rivers from 1985 to 1987 (Fig. 1). These four systems represent two rivers at the extremes of the species' range and two rivers in geographical proximity in the center of the shad's Atlantic distribution.

Ovaries were excised from freshly killed fish and were shipped on ice to our laboratory. Mitochondrial extraction procedures were initiated within 2 d of capture. Briefly, mitochondria were prepared by multiple rounds of differential centrifugation and were further purified by ultracentrifugation on a preformed sucrose step gradient. Mitochondria were lysed to release mtDNA, which was further purified by three rounds of cesium chloride density gradient ultracentrifugation, butanol extraction, dialysis, alcohol precipitation, air drying, and mtDNA resuspension in buffer (for details see Wirgin 1987).

mtDNA Analysis

The restriction enzymes used in this survey were *Ava* II, *Dde* I, *Eco* RI, *Hinf* I, and *Rsa* I. Two other restriction enzymes, *Ava* I and *Bgl* I, produced monomorphic fragment profiles when tested on more than 50 samples and were not used further. Digests were performed according to manufacturer's recommendations (Boerhinger Mannheim, Inc.), with digest times generally ranging from 4 to 6 h. Exact mtDNA concentrations were not determined, although we estimate that <20 ng of mtDNA was used in each digest. The large fragment (Klenow) of DNA polymerase I was used to end-label the 3′ termini of the digested DNA fragments with a cocktail of all four [^{32}P] deoxynucleoside triphosphate precursors at 15°C (Maniatis et al. 1982). Labeled mtDNA fragments were ethanol precipitated (100 and 70%), vacuum dried, and resuspended in 1 × TBE buffer (8.9 mM Tris, 8.9 mM boric acid, 25 mM Na_2EDTA, pH 8.3) and stop load (50% glycerol, 0.25% bromphenol blue, 0.25% xylene cyanole).

Fragments generated by four-and-five-base restriction endonuclease digest varied sufficiently in size that their visualization demanded the use of both agarose and polyacrylamide gels. Large mtDNA fragments (>500 base pairs (bp) were resolved on agarose gels (1–1.8%) made in TBE buffer. Polyacrylamide gels (6%) also made in TBE buffer allowed for the visualization of smaller fragments due to their more restrictive porosity. A 1 kilobase pair (kbp) ladder (Bethesda Research Laboratories) and/or Lambda DNA digested with *Hind* III or with *Hind* III and *Eco* RI were used as molecular weight markers. All gels were dried under vacuum, and fragments were visualized by autoradiography with (at −80°C) and without intensifying screens (at room temperature) using Kodak XAR 5 film. Laser densitometry (LKB Ultroscan) was performed to identify some comigrating bands.

Digest patterns generated from single enzymes were generally identified by a single letter. Due to the large number of polymorphic fragments which were generated by *Dde* I and *Hinf* I digestions, separate letter designations were assigned for *Dde* I and *Hinf* I genotypes scored on agarose and polyacry-

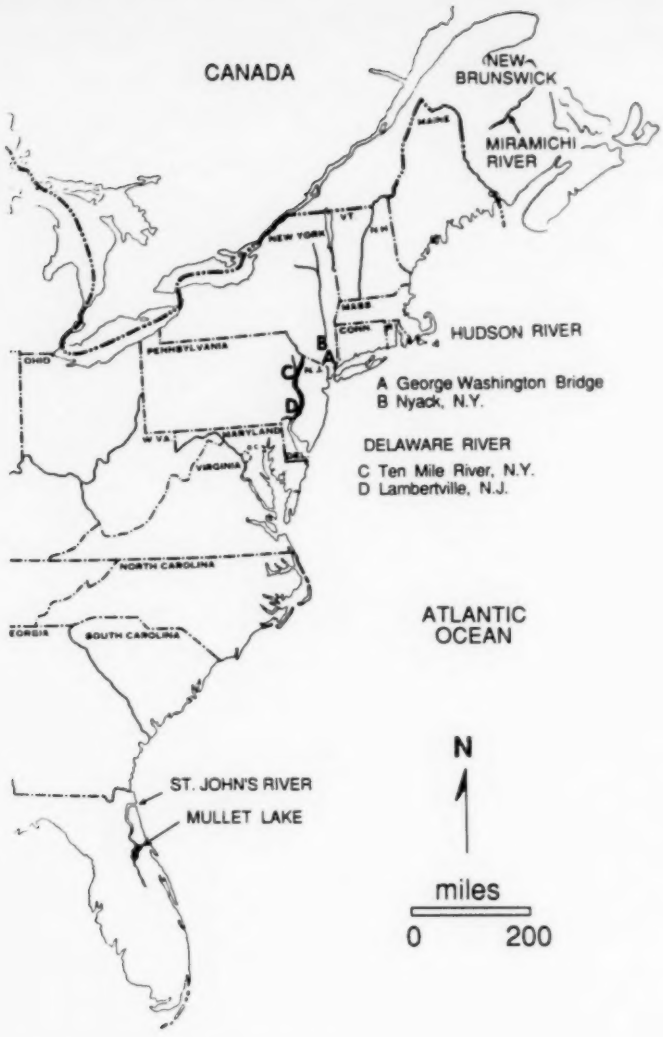

FIG. 1. Map of the Atlantic coast of North America depicting the four collection sites of American shad (1 mile = 1.6 km).

lamide gels. Additionally, multiple letter designations were assigned to *Dde* I genotypes scored on polyacrylamide gels. This was done to show the commonality of a single DNA fragment within a spawning system. For all enzymes, the A genotype was the most frequent among all rivers tested. Each fish was also assigned a multiletter composite genotype based on the results of all individual restriction enzyme digests. The Kolmogorov–Smirnov and the chi square tests were used to evaluate the significance of differences in mtDNA genotypes among spawning systems. In addition, the *F* statistic (Upholt 1977) was used to quantify the extent of mtDNA sequence similarity among individuals, within spawning systems, and between systems. An estimate of restriction pattern diversity (h) within spawning systems was calculated (Nei and Tajima 1981).

Results

The five informative enzymes used in this survey produced 151 restriction fragments, representative of 730 nucleotides, or 4.0% of the approximately 18 300-bp shad mtDNA molecule (as reported by Bentzen et al. 1988). These are not all the fragments generated by these enzymes. Mitochondrial DNA fragments <80 bp were not scored. In addition, comigrating bands (determined by densitometry) were frequently generated, but these were only scored as single fragments. Characterization of the mtDNA fragment number and sizes for each restriction enzyme used in this study for a single American shad from the Hudson River is shown in Table 1. Frequencies of individual restriction enzyme digest patterns (*Ava* II, *Dde* I (agarose and polyacrylamide), *Eco* RI, *Hinf* I (agarose and polyacrylamide), and *Rsa* I) (Table 2) and composite genotypes were compiled for each of the four spawning rivers (Table 3). We were unable to resolve digest patterns for all the enzymes on all samples, and thus, composite genotypes are reported for only 81 of the samples. In total, 57 different composite mtDNA genotypes were detected. In our analysis of stock differences,

TABLE 1. Fragment sizes (base pairs) for an American shad representative of genotype A for each restriction enzyme used in this study.

Band	Restriction enzymes						
	Ava I	*Ava* II	*Bgl* I	*Eco* RI	*Dde* I	*Hinf* I	*Rsa* I[a]
1	12 575	2370	8715	9975	1600	1350	525
2	2 815	2135	3410	7315	810	1050	495
3	1 560	1870	2830	1380	810	1030	460
4	1 250	1720	1825		735	1010	460
5	915	1680	1330		650	790	410
6	415	1460			595	740	410
7		1200			540	710	400
8		960			520	680	395
9		865			515	640	395
10		530			510	530	355
11		425			490	515	325
12		415			480	500	305
13		400			455	480	285
14		380			455	470	280
15		350			420	430	275
16		325			395	430	260
17		300			350	385	240
18		285			330	380	240
19		260			315	365	225
20		250			295	355	205
21					295	310	200
22					285	295	180
23					285	265	170
24					270	230	120
25					270	215	90
26					245	185	
27					230	175	
28					200	165	
29					200	160	
30					190	155	
31					175	130	
32					175	110	
33					170	100	
34					160	90	
35					155	80	
36					150		
37					135		
38					135		
39					95		
40					90		
41					75		
42					70		

[a]Only data from *Rsa* I digests visualized on polyacrylamide gels are presented.

TABLE 2. Frequencies of individual restriction enzyme mtDNA genotypes in American shad for four spawning rivers.

*Ava*II *genotypes (n = 109)*

River	A	B	C	D	E	F	G	H	I	J
St. John's	11	15	3	0	0	1	2	0	1	1
Delaware	11	12	0	0	0	0	1	1	0	0
Hudson	21	12	0	0	1	0	1	0	0	0
Miramichi	7	7	0	1	0	0	0	0	0	0

Dde I *genotypes (agarose) (n = 109)*

	A	B	C	D
St. John's	12	22	0	1
Delaware	17	7	2	0
Hudson	22	11	0	0
Miramichi	11	0	4	0

Dde I *genotypes (polyacrylamide) (n = 102)*

	A	B	C	D	E	F	J	BC	BG	BGF	DE	EF
St. John's	25	1	0	1	4	0	1	0	0	0	2	0
Delaware	18	0	3	0	1	0	0	2	0	0	0	0
Hudson	26	3	1	0	0	0	0	0	1	0	0	0
Miramichi	4	0	1	0	0	6	0	0	0	1	0	1

Eco RI *genotypes (n = 119)*

	A	B
St. John's	27	10
Delaware	28	0
Hudson	38	1
Miramichi	15	0

Hinf I *genotypes (agarose) (n = 93)*

	A	B
St. John's	25	8
Delaware	10	12
Hudson	18	8
Miramichi	7	5

Hinf I *genotypes (polyacrylamide) (n = 114)*

	A	B
St. John's	36	1
Delaware	19	8
Hudson	29	6
Miramichi	15	0

Rsa I *genotypes (n = 108)*[a]

	A	B	C	D	A(B)	A(C)	B(C)	B(D)	E	F
St. John's	4	6	17	1	1	0	4	1	0	0
Delaware	14	6	1	0	1	2	0	0	0	1
Hudson	21	7	4	0	1	1	0	0	0	0
Miramichi	11	1	0	0	0	0	0	0	3	0

[a]Letters in parentheses indicate heteroplasmy for that genotype.

we have considered the Hudson and Delaware both as individual stocks and as a joint stock.

We scored several different types of mtDNA polymorphisms. One form of variation was a definitive restriction site polymorphism, as revealed by *Eco* RI, in which the number of fragments varied among individuals and in which the sum of the molecular size (base pairs) of two smaller fragments was equal to that of a larger polymorphic fragment. The second class of variants, as generated by *Ava* II, *Dde* I, *Hinf* I, and *Rsa* I, was characterized by differences in the electrophoretic mobility of individual fragments. These were most frequently detected on polyacrylamide gels. It is possible that this variation represented small differences in the overall length of the shad mtDNA molecule which can be most easily visualized in small DNA fragments. Another plausible explanation is that they represented base substitutions within the mtDNA molecules at other than restriction enzyme recognition sites.

Ten mtDNA genotypes were revealed with *Ava* II. Polymorphisms were primarily observed within two molecular size ranges: 1200–2000 and 250–800 bp. In both cases, differences were detected in the electrophoretic mobility of individual fragments and the absence of single fragments. For example, genotypes A and B differed in the electrophoretic mobility of an approximately 1400-bp fragment. In addition, genotype C was

TABLE 3. Composite mtDNA genotypes for American shad from four spawning rivers.

Composite number	Composite genotype[a]	River			
		St. John's	Delaware	Hudson	Miramichi
1	AAAAAAA	0	2	3	1
2	AAAAAAB	1	1	1	0
3	AAAAAAC	0	1	0	0
4	AAAABAA	0	1	3	0
5	AAAABAA(B)	0	0	1	0
6	AAAABAB	0	1	0	0
7	AABAAAA	0	0	1	0
8	AA(BG)ABAB	0	0	1	0
9	AA(EF)ABAA	0	0	0	1
10	AAFAAAE	0	0	0	2
11	ABAAAAA	1	0	1	0
12	ABAAAAB	1	0	0	0
13	ABAAABA	0	1	0	0
14	ABAAABA(B)	0	1	0	0
15	ABAAABB	0	1	2	0
16	ABAABAA(C)	0	1	0	0
17	ABABAAB	1	0	0	0
18	ABABAAB(D)	1	0	0	0
19	ABABAAC	1	0	0	0
20	ABABA(B)AA	0	0	1	0
21	ABABBAA	1	0	0	0
22	AB(BC)AAAA	0	1	0	0
23	ABEAAAB	1	0	0	0
24	ACCAAAB	0	0	0	1
25	ADAABAD	1	0	0	0
26	BAAAAAA	0	0	3	1
27	BAAAABB(C)	1	0	0	0
28	BAAABAA	0	3	1	1
29	BAAABAC	0	0	1	0
30	BAABBAA(B)	1	0	0	0
31	BA(BGF)AAAA	0	0	0	1
32	BACAAAC	1	0	0	0
33	BACABAB	0	1	0	0
34	BA(DE)AAAB(C)	1	0	0	0
35	BAFABAA	0	0	0	1
36	BBAAAAA	0	0	1	0
37	BBAAAAC	4	0	0	0
38	BBAABAB	0	0	1	0
39	BBAABBF	0	1	0	0
40	BBABAAB(C)	1	0	0	0
41	BBABAAC	1	0	0	0
42	BBBABAA	0	0	1	0
43	BBEABAC	1	0	0	0
44	BBEBAAC	1	0	0	0
45	BBGABAB(C)	1	0	0	0
46	BCFABAE	0	0	0	1
47	CADAAAC	1	0	0	0
48	CA(DE)AAAB	1	0	0	0
49	CAEAAAA	1	0	0	0
50	EBAAABA	0	0	1	0
51	FBAAAAA	1	0	0	0
52	GABAAAC	0	0	1	0
53	GBAAAAC	1	0	0	0
54	GBAABAC	1	0	0	0
55	HCCAAAA	0	1	0	0
56	IAAABAC	1	0	0	0
57	JAAAAAC	1	0	0	0
Total number of samples		30	17	24	10
Total number of genotypes within river		27	14	17	9
Total number of genotypes unique to that specific river		25	9	10	6

[a]Letters from left to right indicate mtDNA genotypes for *Ava* II, *Dde* I (agarose), *Dde* I (polyacrylamide), *Eco* RI, *Hinf* I (agarose), *Hinf* I (polyacrylamide), and *Rsa* I (polyacrylamide). Single letters in parentheses indicate heteroplasmy for the preceding restriction enzyme. Multiple letters in parentheses indicate *Rsa* I (polyacrylamide) genotype.

missing a 1200-bp fragment that was present in all other genotypes. However, this genotype exhibited a 1510-bp fragment not evident in other genotypes. We also scored differences among smaller mtDNA fragments whose molecular size ranged between 250 and 350 bp and which were separated on 6% polyacrylamide gels. For example, genotype C revealed comigrating bands of 325 bp, while genotypes A, B, D, and E exhibited 350-bp fragments. Genotype D was missing a 280-bp fragment present in all other genotypes. Significant differences were not found in the frequencies of *Ava* II genotypes among any of the rivers tested.

We were able to differentiate four *Dde* I diget patterns on agarose gels. Genotype A was characterized by the presence of a 540-bp fragment not seen in the B genotype. Instead, genotype B displayed a 1015-bp fragment (Fig. 2). The frequency of genotype B decreased with latitude and was absent from our Miramichi collection. For example, this genotype was found in 63% of St. John's fish, 31% of Hudson–Delaware fish, and was totally absent from our Miramichi collection. Genotype C was characterized by a 1430-bp fragment and was missing two fragments, 610 and 595 bp, that were present in the other two genotypes. Genotype C was only found in six fish, four from the Miramichi River (27%) and two from the Delaware. Frequencies of *Dde* I generated genotypes differed significantly between the St. John's and Hudson–Delaware samples (max $D = 0.31$, $P \leq 0.05$).

We observed 12 different *Dde* I genotypes on 6% polyacrylamide gels. Based on molecular sizing, these are different mtDNA fragments than were scored on agarose gels. Variation was seen in the electrophoretic mobility of fragments which ranged from about 250 to 550 bp (Fig. 3). Six of 12 Miramichi fish exhibited a genotype F not seen in any other fish. A significant difference in *Dde* I polyacrylamide mtDNA genotypes was observed between our Miramichi and Hudson–Delaware samples (max $D = 0.57$, $P \leq 0.01$).

Eco RI revealed two genotypes resulting from a restriction site polymorphism. Genotype A was characterized by three fragments on agarose gels: 9970, 7315, and 1370 bp. Genotype B was characterized by four fragments; the largest mtDNA fragment (9970 bp) was cleaved into two smaller mtDNA fragments of approximately 5100 and 4220 bp. Genotype A was found in fish from all rivers, but genotype B was found only in nine fish, eight from the St. John's and one from the Hudson. The St. John's River stock could be distinguished from all other stocks by *Eco* RI generated genotypes (St. John's versus Hudson–Delaware, $\chi^2 = 10.8$, $P < 0.001$; St. John's – Miramichi, $\chi^2 = 3.703$, $P \leq 0.05$).

Separation of *Hinf* I digests on polyacrylamide gels revealed two genotypes (Fig. 4). Genotype A lacked a 223-bp fragment that was found in genotype B, while genotype B lacked a 185-bp fragment that was present in genotype A. The St. Johns exhibited a low frequency of genotype B (3%), and this genotype was totally absent in our Miramichi sample. However, our Hudson–Delaware collection exhibited this genotype in a moderate frequency (23%). Hudson–Delaware and Miramichi samples could be distinguished by *Hinf* I generated mtDNA genotypes ($\chi^2 = 4.1$, $P \leq 0.05$). Two different genotypes were distinguishable in *Hinf* I digests resolved on agarose gels, but we could not delineate stocks based on data from *Hinf* I agarose gels.

Rsa I digestions produced 10 different genotypes on polyacrylamide gels. Variation was seen in fragments which ranged in size from 300 to 365 bp. Variation in the electrophoretic

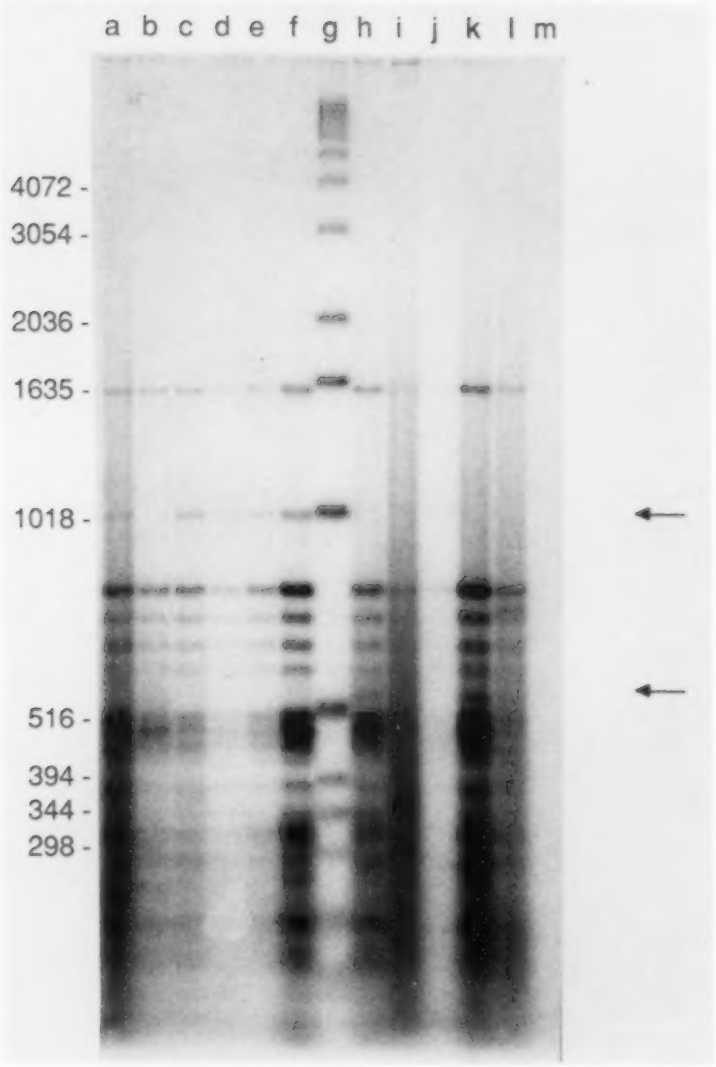

FIG. 2. Autoradiograph of *Dde* I digests of American shad mtDNA separated on a 1.6% agarose gel. Polymorphism can be seen in the presence/absence of 1015- and 540-bp fragments as indicated by the two arrows. Representatives of genotype A can be seen in lanes b, h, i, k, and l, while samples in lanes a, c, d, e, f, and j depict genotype B. The molecular weight standard, a 1-kbp ladder, is in lane g. Molecular sizes (bp) are indicated on the left.

mobility of these small fragments could be attributed to differences of 10–15 bp in the size of the polymorphic fragments (Fig. 5). Heteroplasmy for electrophoretic mobility variants was evident in 11 individuals. Three different mtDNA fragments were detected as the heteroplasmic mtDNA fragment in these individuals. These same individuals were not heteroplasmic using other restriction enzymes. Genotype A was the most abundant in the Delaware, Hudson, and Miramichi rivers (56–73%), while only four (12%) of our St. John's samples exhibited this genotype. Genotype C was the most common (50%) in the St. John's. Genotype E was found only in three fish, all from the Miramichi (20%). Frequencies of *Rsa* I fragments differed significantly between the St. John's and Hudson–Delaware (max $D = 0.52$, $P \leq 0.01$) and the St. John's and Miramichi (max $D = 0.61$, $P \leq 0.01$).

The number of shared fragments (F value) between each pair of mtDNA genotypes was determined (Upholt 1977). These F values were weighted based on the number of base pairs each class of restriction enzymes contributed to the total number of base pairs scored. We did not convert these F values to estimates of base sequence divergence (p) because we could not definitively attribute most polymorphisms to single base substitutions, an assumption upon which this statistic is founded (Nei and Li 1979). The F values ranged from 0.887 to 0.997

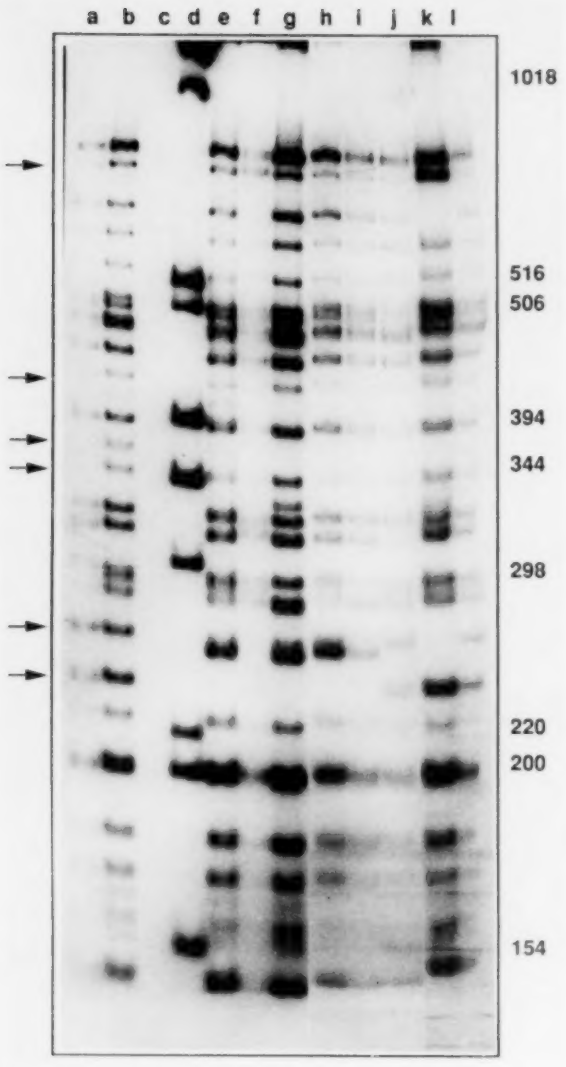

FIG. 3. Autoradiograph of *Dde* I digests fractionated on a 6% polyacrylamide gel. Six different mtDNA genotypes can be seen. Polymorphic regions are indicated by the arrows. Lane to genotype designations are as follows: a, genotype A; b, genotype BG; c, no sample; d, molecular weight standard (1-kbp ladder); e, genotype BGF; f, genotype F; g, genotype EF; h, genotype F; i, genotype F; j, genotype A; k, genotype C; l, genotype A.

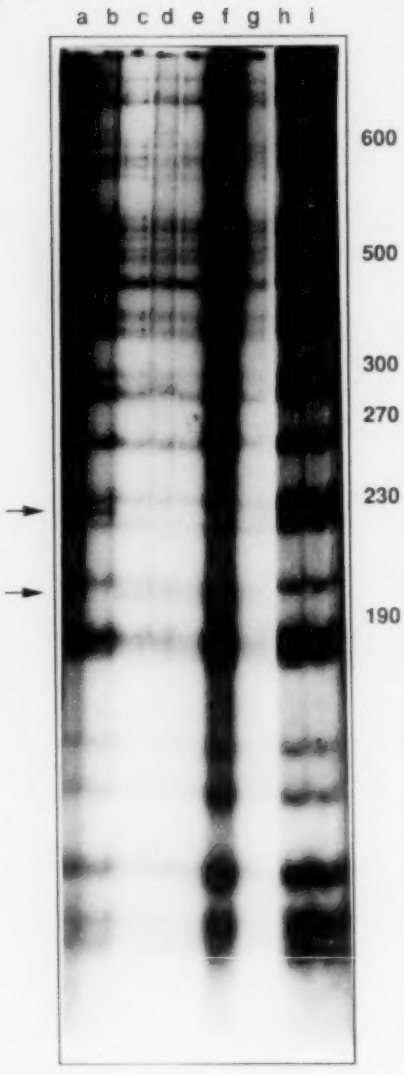

FIG. 4. Autoradiograph of *Hinf* I digested shad mtDNA separated on a 6% polyacrylamide gel. Variation can be seen in the presence/absence of 223- and 185-bp fragments. Lanes a, c, d, e, f, and g depict genotype A, while lanes b, h, and i have genotype B.

between pairs of individuals. Mean intraspawning system F values ranged from 0.945 (Miramichi) to 0.956 (Delaware). Our estimate of restriction pattern diversity (Nei and Tajima 1981) based on the number of samples analyzed and the number and frequency of composite genotypes in individual spawning systems was highest in the St. John's (h = 0.975) and lowest in the Hudson River (h = 0.885). The Miramichi and Delaware rivers presented intermediate values of 0.946 and 0.929, respectively.

In total, we detected 57 different composite genotypes among 81 shad tested. The St. John's collection revealed the largest number of different composite genotypes (27). The St. John's also harbored the largest number of fish, with unique composite genotypes (24). Thus, 80% of St. John's fish exhibited composite genotypes not seen in other rivers. A smaller percentage of fish from more northern rivers displayed unique genotypes (42–60%). There was no sharing of any composite genotypes among all four rivers, and in fact, only six of the composite genotypes were observed in more than one river. Most importantly, the frequency of individual composite genotypes never exceeded 15% in any river.

Discussion

Identification of Stocks

We consider stock identification to encompass two related, but separate questions requiring differing degrees of resolution

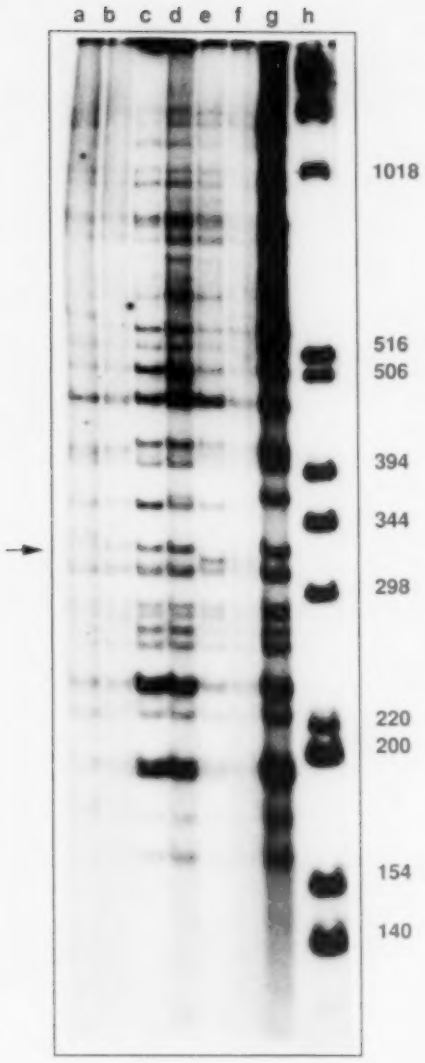

FIG. 5. Representative *Rsa* I digests of shad mtDNA fractionated on a 6% polyacrylamide gel. Polymorphisms can be observed in the electrophoretic mobility of several mtDNA fragments which range from approximately 300 to 365 bp. Polymorphic region is indicated by the arrow. The molecular weight standard 1-kbp ladder is in lane h, genotype A is seen in lanes b, c, and d, genotype B is in lane g, genotype C is in lane a, and genotype E in lanes e and f.

to address. First, can separate stocks be identified? This task requires the demonstration of significant differences in the frequency of a single or composite polymorphic markers. An affirmative finding presents the opportunity to manage the fishery as separate units. A second and more demanding task, although not routinely addressed, is whether a reasonable percentage of fish from these defined stocks can be uniquely assigned an origin in a mixed fishery. The immediate application of this information lies in estimating the relative contribution of the defined stocks to the mixed fishery. Frequently, as in the case of shad, Pacific salmon, or striped bass, fishing mortality may be greatest at sites far removed from natal estuaries. The identification of ancestry of individuals or contingents of fish in a mixed fishery may dictate the demonstration of individual stock-specific markers that are minimally at a moderate frequency within the previously defined stocks or the detection of a suite of polymorphic markers, each of which differs in frequency among the stocks tested. If an excessively high level of variability is detected within spawning systems, individual stocks can still be defined, but the identification of ancestry of individual fish in a mixed fishery becomes more complex.

We tested the hypothesis that geographically separate shad spawning rivers contained discrete populations. Implicit to this study was the assumption that spawning fish collected at spawning sites could be considered natal to that system. Mark–recapture studies have shown that American shad are relatively faithful homers (about 97%) (Hollis 1948; Mansuetti and Kolb 1953; Williams and Daborn 1984; Melvin et al. 1986). Meristic data have suggeted that shad not only home to their natal rivers but also to tributaries within some of the major northern spawning rivers (Carscadden and Leggett 1975). However, it has been demonstrated that even low levels of infidelity may be sufficient to prevent the establishment of river-specific genotypic frequencies (Allendorf 1983). For example, Lewontin (1974) suggested that a migration rate as small as one breeding individual in a thousand per generation is sufficient to prevent genetic differentiation of diploid characters in moderate-sized populations.

Unlike many studies of mtDNA genotypes in terrestrial or freshwater fauna, we did not detect fixed single restriction enzyme or composite mtDNA genotypes in any shad spawning system. We did find several single enzyme generated mtDNA genotypes which were restricted to a single river and which were found in a moderate frequency within that system. Frequency differences in single restriction enzyme generated mtDNA genotypes were also apparent. Our data support the hypothesis that the Miramichi, Hudson–Delaware, and St. John's rivers support separate stocks of American shad. Bentzen et al. (1989) also detected significant differences in mtDNA genotype frequencies among shad from Gulf of St. Lawrence drainages, such as the Miramichi, and other rivers. We were unable to demonstrate significant differences between Hudson River and Delaware River shad aggregations.

Our findings suggest an absence of substantial gene flow among shad spawning systems which are geographically distant. Based on the demonstration of significant mtDNA frequency differences among these populations, female shad exhibit accurate region-specific homing behavior. Given the maternal inheritance of mtDNA genotypes, we are unable to assess the fidelity of male homing behavior. Our inability to distinguish Hudson from Delaware shad may be interpreted in two different ways. First, female homing infidelity between these geographically proximal systems may be currently, or in the recent evolutionary past, substantial. An equally plausible explanation is that gene flow between these two systems is currently minimal, but insufficient time has elapsed since their division for the buildup of genotypic frequency differences. Given that the impact of the most recent glacial event was only about 18 000 yr ago (Fodor 1981), only slight differences in mtDNA genotypic frequencies might be anticipated between these two systems. For example, if we apply a rate of mtDNA change of 0.02 per base pair per million years (Brown et al. 1979), then a diversity value of approximately $p = 0.0004$

would be projected when comparing Hudson and Delaware shad. In fact, we have reported maximum mtDNA sequence diversity between striped bass from the same geographic area (Hudson River and Chesapeake Bay) as $p = 0.0004$, a value which is consistent with these estimates (Wirgin et al. 1989b). It is possible that the use of additional restriction endonucleases or the analysis of more Hudson and Delaware samples may reveal additional informative polymorphisms, and thus it would be premature to consider these two spawning systems as a single stock.

It may be postulated that spawning populations or "stocks" of shad could represent geographic regions rather than specific rivers. Williams (1985) delineated shad into three distinct geographic spawning groups based on otolith analysis, Melvin (1984) characterized three separate stocks of shad based on multiple meristic and morphometric characters, and Dadswell et al. (1987) found three distinct oceanic aggregates of shad within the overall mixed Atlantic coastal fishery. Our data minimally support this division, although analysis of mtDNA genotypes of more fish from these rivers and samples from additional mid-Atlantic spawning systems may enable us to further delineate additional shad stocks.

Characterization of mtDNA Diversity and Its Geographic Distribution among Shad

We detected only one definitive restriction enzyme site polymorphism among spawning systems which included two rivers at the extremes of the shad's Atlantic distribution. Using a battery of different six-base-cutting restriction enzymes, Bentzen et al. (1989) also reported low levels of overall mtDNA sequence diversity in American shad ($p = 0.0037$). These results are consistent with work by Chapman (1987, 1989) and Wirgin et al. (1989a, 1989b) on sympatric populations of striped bass from many of the same spawning rivers. We have also seen a total absence of mtDNA diversity among anadromous Atlantic tomcod (*Microgadus tomcod*) from three spawning rivers within this same Northwest Atlantic area (Wirgin et al. 1990). Despite the common impact of glaciation, somewhat higher levels of mtDNA sequence diversity were reported in populations of anadromous cisco (*Coregonus artedii*) from the James–Hudson bays (mean $p = 0.0052$) (Bernatchez and Dodson 1990). These workers hypothesized that postglacial colonization of these systems by cisco from genetically divergent refugia may account for the current moderate levels of mtDNA diversity. The lack of mtDNA diversity in extant shad populations points to genetic monomorphism among postglacial founders of these stocks or extreme population bottlenecks in the recent evolutionary past.

A single definitive restriction site polymorphism was primarily seen in St. John's samples (26%) tested with *Eco* RI. One individual from the Hudson also displayed this genotype which may have represented straying of a St. John's fish, or alternatively, the prevalence of this genotype in several populations prior to their isolation. We have seen this polymorphism in shad from the St. John's over 3 yr of sampling and we believe it may be used to identify St. John's fish in a mixed sample.

We detected a large number of mtDNA fragments that exhibited differing electrophoretic mobility, primarily on polyacrylamide gels. These variants were often river or region specific and proved valuable in stock discrimination. It is possible that these represent minor size variants due to differential copy numbers of short repetitive mtDNA sequences which may only be resolved in relatively small fragments. Support for this hypothesis comes from the earlier results of Bentzen et al. (1988) who detected multiple copies of short (<40 bp) repetitive mtDNA fragments in shad which probably mapped to the D-loop region. Both Densmore et al. (1985) and Wirgin et al. (1989b) observed small mtDNA fragments in lizards and striped bass, respectively, which differed in electrophoretic mobility in polyacrylamide gels, although the molecular basis for this variation was not characterized. We did not see concordance in molecular weight differences among these mtDNA samples when using a battery of different restriction endonucleases, an approach which can be used to unequivocally identify large mtDNA length variants (Avise et al. 1989). However, given the small size difference among these polymorphic mtDNA fragments, and the potential for these variations to go undetected in moderate to large mtDNA fragments, these results are not unexpected. It should also be cautioned that in the event that the electrophoretic mobility variants that we scored are indeed minor size variants that are detectable using only some restriction enzymes, the single-enzyme genotypes that we presented may not be independent of each other. Non-independence of genotypes could lead to inflated genotype diversity values.

We cannot disregard the hypothesis that some of these polymorphic fragments harbor base substitutions that alter the conformation of these mtDNA fragments sufficiently to affect their electrophoretic mobility. Singh et al. (1987) also reported small human mtDNA fragments which differed in electrophoretic mobility on polyacrylamide gels. Cloning of these fragments and sequence analysis revealed that these human mtDNA fragments contained nonrestriction site base substitutions.

Hinf I (polyacrylamide) genotype B was most common in the center of the shad's distribution and was either present in very low frequencies or totally absent at the extremes of the shad's distribution. One St. John's sample and none of the Miramichi samples displayed this genotype. Bentzen et al. (1989) also reported an *Eco* RV genotype in the center of shad's range in moderate frequency but its total absence at the extremes of the range. In contrast, *Rsa* I and *Dde* I revealed clines in mtDNA genotype frequencies. For example, the frequency of *Rsa* I, genotype A increased directly with latitude from 12% in the St. John's to 73% in the Miramichi. *Dde* I (agarose) also revealed a cline, with the frequency of genotype A increasing with latitude. Bentzen et al. (1989) also reported a cline in the frequency of mtDNA length genotypes, with the largest mtDNA length genotype only found at the southern end of the shad's range. These clines in mtDNA genotype frequencies could result from founder effects of shad from ancestral populations and therefore may be used to track the overall historical movement of shad populations.

Assuming that ancestral shad populations were restricted to the southeastern United States during glacial maxima, these older southern populations would be expected to exhibit more variability because they have had additional time to undergo DNA alterations. The St. John's had the highest restriction pattern diversity value, $h = 0.975$, as well as the largest number of genotypes unique to a single river. In addressing a closely aligned but different issue, Bentzen and co-workers (1989) found the heterogeneity of mtDNA genotype frequencies to be greatest among rivers north of the Delaware. They attributed this finding to founder effects resulting from postglacial colonization of these rivers from southern refugia. Other data support this finding in that we found greatest diversity in the south-

ernmost river, one which could have served as a refugium during glaciation.

We detected 57 different composite genotypes out of 81 shad tested. Almost all of this diversity resulted from the presence of electrophoretic mobility variants. Avise et al. (1989) also reported numerous composite mtDNA genotypes resulting from both restriction site polymorphisms and presumptive length variants when screening two collections of the marine clupeid fish, menhaden (*Brevoortia tyrannus/patronus*). Thirty-two of 33 fish exhibited individual-specific composite mtDNA genotypes when tested with 18 five-and-six base-cutting restriction endonucleases. These workers invoked the enormous effective population size of menhaden ($>10^8$) as a likely explanation for this extensive polymorphism. Kornfield and Bogdanowicz (1987) also reported substantial levels of mtDNA base sequence diversity and an absence of mtDNA length variants in another species of marine clupeid fish, the Atlantic herring (*Clupea harengus*). Current estimates of population size of Atlantic herring are also very large which may in part account for their substantial levels of base sequence diversity. Approximately 70% of the American shad that we screened exhibited unique composite mtDNA genotypes, although very little of this hypervariability could be attributed to restriction site polymorphisms. The menhaden and herring data suggest a rapid rate of mtDNA base sequence change in the clupeid fish, which is probably comparable with that observed in primates and other animals (Brown et al. 1979). Our finding of a lack of base sequence diversity in American shad, in combination with extensive electrophoretic mobility polymorphisms, suggests a rapid rate and recent generation of the latter form of variants.

By considering composite genotypes, 80% of St. John's samples, 53% from the Delaware, 42% from the Hudson, and 60% of Miramichi shad exhibited unique composite genotypes. The presence of these unique composite genotypes reinforces the hypothesis of genetic isolation among these populations. However, given our small sample size and the large number of composite genotypes per river, it is possible that we missed many commonly shared composite genotypes. It is also possible that many of these unique composite genotypes are found in other intervening rivers not sampled in this study. The value of these composite genotypes in stock identification of unknown fish from the mixed coastal fishery is minimal given the low frequency of individual unique genotypes in the various rivers. For example, the highest frequency of any unique composite genotype in a single river is less than 15% of the samples from that river.

The use of mtDNA is not a panacea for all stock identification problems, yet in certain cases this approach may provide valuable discriminatory markers. The compilation of multienzyme generated composite mtDNA genotypes may be impractical for the identification of ancestry of large numbers of samples collected in a mixed fishery due to excessive cost and time. Additionally, unique composite genotypes are frequently in very low frequencies within individual stocks.

Single enzyme digests of mtDNA in terrestrial and freshwater comparisons frequently reveal fixed alternative polymorphic genotypes; however, such differences have not been detected in Northwest Atlantic anadromous (Chapman 1987, 1989; Wirgin et al. 1989a, 1989b), catadromous (Avise et al. 1986), and marine fishes (Kornfield and Bogdanowicz 1987). In some instances, population-specific, single enzyme generated mtDNA genotypes are observed in a moderate frequency, thereby permitting the unambiguous identification of ancestry of these individuals in a mixed fishery. In this study, we have detected population-specific *Rsa* I, *Dde* I, and *Eco* RI genotypes which may allow for discrimination of these individuals in mixed fisheries. However, in the absence of data on more intervening rivers, it is possible that these mtDNA genotypes may be region rather than river specific. Additionally, Bentzen et al. (1989) reported *Hae* II and *Eco* RV mtDNA genotypes that were informative in identifying representatives of different shad spawning systems. In total, these results suggest that some single enzyme or two enzyme generated mtDNA genotypes may be used to uniquely identify representatives of some American shad stocks. If these stocks make substantial contributions to a mixed fishery, this information could prove informative to address the question of relative contribution. These markers may be particularly useful when dealing with mixed fisheries such as shad to which so many different spawning stocks contribute.

Acknowledgements

We thank Rick Long, Terry Cheek, Lawson Snyder, Fred Lewis, the New Paltz NYDEC, Jon Punola, Bob Gabrielson, Ron Ingold, Everett Nack, and Bob Randall for help in obtaining the shad, Rui Proenca, Su Chen, Yi Jun Yang, and Dick Conley for their assistance in processing and conducting electrophoresis on the samples, and Dr. R. Nicholas Hoffmann for computer assistance. Dr. John Waldman provided constructive criticisms on several drafts of this manuscript. This research was supported by grants 666281 and 667145 to J. G. from the PSC-CUNY Research Award Program of the City University of New York.

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Some Factors Regulating Piscivory of Brook Trout[1], *Salvelinus fontinalis*, in Lakes of the Laurentian Shield

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East, P., and P. Magnan. 1991. Some factors regulating piscivory of brook trout, *Salvelinus fontinalis*, in lakes of the Laurentian Shield. Can. J. Fish. Aquat. Sci. 48: 1735–1743.

A survey of 13 lakes containing brook trout, *Salvelinus fontinalis*, and northern redbelly dace, *Phoxinus eos*, five lakes containing trout and creek chub, *Semotilus atromaculatus*, and six lakes containing trout, dace, and chub indicated that prey-fish could represent up to 30% of trout diet by weight. We observed that trout preyed almost exclusively on dace, predation increased with trout size, predation on dace was significantly higher in the *Salvelinus-Phoxinus-Semotilus* association than in the *Salvelinus-Phoxinus* association even though trout were significantly smaller in the former than in the latter association, and predation in the *Salvelinus-Phoxinus-Semotilus* association was higher in two lakes and nearly always absent in the other four. Laboratory experiments indicated that small trout (150–250 mm total length (TL)) preferred small prey-fish (40–60 mm TL), large trout (250–380 mm TL) showed no significant preference with regard to the size of prey-fish (up to 170 mm TL), trout of both size classes preferred dace when dace and chub were present, presence of refuge (*Cassandra calyculata*) for prey-fish significantly reduced the number of attacks and captures upon dace, and large trout switched from an active to a sit-and-wait foraging pattern when a prey refuge was present.

Un inventaire de 13 lacs contenant de l'Omble de fontaine, *Salvelinus fontinalis*, et du Ventre rouge du Nord, *Phoxinus eos*, cinq lacs contenant de l'Omble et du Mulet à cornes, *Semotilus atromaculatus*, et six lacs contenant les trois espèces ensemble indique que les poissons-proies peuvent constituer jusqu'à 30% de la diète des ombles en poids. Nous avons observé que les ombles effectuaient une prédation presqu'exclusivement sur les ventres rouges, que la prédation augmentait en fonction de la taille des ombles, que la prédation sur les ventres rouges était significativement plus élevée dans l'association *Salvelinus–Phoxinus–Semotilus* que dans l'association *Salvelinus–Phoxinus* en dépit du fait que les ombles étaient significativement plus petits dans la première association de poissons que dans la dernière et que la prédation dans l'association *Salvelinus–Phoxinus–Semotilus* était plus élevée dans deux lacs et presque nulle dans les quatre autres. Des expériences en laboratoire ont indiqué que les petits ombles (150–250 mm longueur totale (LT)) avaient une préférence significative pour les poissons-proies de petite taille (40–60 mm LT), que les grands ombles (250–380 mm LT) ne démontraient aucune préférence significative quant à la taille des poissons-proies (jusqu'à 170 mm LT), que les ombles des deux classes de tailles préféraient les ventres rouges aux mulets, que la présence de refuge (*Cassandra calyculata*) pour les poissons-proies diminuait significativement le nombre d'attaques et de captures sur les ventres rouges et que les grands ombles passaient d'un comportement de recherche active à un comportement de chasse à l'affût lorsqu'un refuge était disponible pour les proies.

Received November 20, 1989
Accepted March 13, 1991
(JA366)

Reçu le 20 novembre 1989
Accepté le 13 mars 1991

Piscivory has been investigated from various aspects such as impact of predators on prey populations (Garman and Nielsen 1982; Loftus and Hulsman 1986; Schlosser 1988; Johannes et al. 1989), role of piscivores in structuring fish communities (Robinson and Tonn 1989), size-limited predation (Hargreaves and LeBrasseur 1986; Tonn and Paszkowski 1986), patterns of predation (Keast 1979, 1985; Storck 1986), and behavioral responses of prey to predators (Hobson 1979; Fraser and Emmons 1984; Godin 1986; Pitcher 1986; Tonn and Paszkowski 1987; Rahel and Stein 1988). However, considering that the degree of fish utilization by a given species may vary with life stage, period of the year, and habitat and location (Keast 1979, 1985; Werner et al. 1983; Mittelbach 1986; Dill 1987), very few studies have investigated what factors determine the degree of piscivory of a species.

Brook trout, *Salvelinus fontinalis*, is often found with potential prey-fish in oligotrophic lakes on the Laurentian Shield. These prey-fish are mainly creek chub, *Semotilus atromaculatus*, and northern redbelly dace, *Phoxinus eos*, introduced into the lakes by bait fishermen 30–50 yr ago. Earlier studies carried out in different lakes of this system showed an important variability in fish utilization by trout (Magnan 1988; Lachance and Magnan 1990; Tremblay and Magnan 1991). Brook trout may be considered a generalist feeder (as defined by Keast 1979), eating mostly zoobenthos, zooplankton, terrestrial insects, and fish (see Scott and Crossman 1973; Power 1980; Magnan 1989). This salmonid thus provides a good model to investigate what factors control piscivory in this group of fish.

The first objective of the present study was to examine the feeding habits of brook trout in 24 lakes with different biotic and abiotic conditions (fish communities, morphometry, and habitats) to identify what factors might account for the variability in their piscivorous behavior. The second objective was

[1]The authors prefer the common name "charr" to designate species of the genus *Salvelinus*.

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TABLE 1. General characteristics of the study lakes, grouped by the composition of their fish communities (the number of stomachs analysed is in parentheses).

Lake	Surface area (ha)	Mean total length of brook trout captured ± 1 SD (mm)
	Salvelinus – Phoxinus lakes	
Au Tremble	28.0	262.5 ± 16.9 (29)
Au Sable	47.0	299.1 ± 58.8 (77)
Belette	16.5	283.2 ± 31.5 (82)
Bleu	52.0	261.0 ± 28.5 (26)
Cerné	10.0	245.1 ± 22.9 (27)
De la Ferme	83.0	234.7 ± 19.9 (20)
De la Poche	9.0	284.1 ± 21.5 (79)
Des Joncs	13.0	300.9 ± 44.0 (51)
Du Vautour	8.0	286.1 ± 37.5 (30)
Epervier	8.0	292.2 ± 27.6 (24)
Léonard	13.0	290.1 ± 41.5 (96)
Roméo	10.0	301.2 ± 20.1 (30)
Verso	9.5	292.3 ± 43.9 (22)
Total	23.6 ± 23.1	286.6 ± 38.4 (593)
	Salvelinus – Semotilus lakes	
Gaspard	10.5	226.2 ± 32.5 (39)
Longue-vue	27.5	223.1 ± 24.6 (45)
Marmotte	11.7	249.7 ± 33.6 (52)
Marshall	37.2	225.6 ± 36.4 (41)
Moineaux	28.3	279.4 ± 30.6 (52)
Total	23.4 ± 11.5	248.7 ± 34.8 (229)
	Salvelinus – Phoxinus – Semotilus lakes	
Argent	28.0	183.4 ± 63.3 (5)
Bourassa	75.0	290.3 ± 22.5 (27)
Cutaway	36.0	259.5 ± 35.1 (76)
Du Camp	8.0	270.4 ± 39.4 (77)
French	3.0	253.7 ± 33.2 (73)
Vison	70.0	271.0 ± 26.7 (83)
Total	36.6 ± 30.4	269.3 ± 32.1 (341)

to test in the laboratory specific hypotheses that were raised by the results obtained in the field: H_1: efficiency of predation on prey-fish increases with the size of brook trout; H_2: brook trout is selective with regard to the size of prey-fish; H_3: brook trout is selective with regard to prey species; H_4: presence of refuges decreases brook trout predation on prey-fish.

Materials and Methods

Field Study

The 24 study lakes were located in Mastigouche (46°40′N, 73°20′W) and St-Maurice (47°05′N, 73°20′W) reserves north of Trois-Rivières (Québec) (Table 1). The lakes of this region are typical of small oligotrophic temperate zone lakes with respect to surface area, mean depth, conductivity, dissolved oxygen, thermal stratification, and Secchi disk transparency (see Magnan 1982, 1988). Because the study lakes were within a 40-km radius, they were exposed to similar climatic conditions. These lakes are subject to sportfishing for brook trout, and exploitation is carefully controlled by the Québec government (Magnan 1988).

The lakes were divided into three groups referred to hereafter as "*Salvelinus–Phoxinus* lakes" containing brook trout and northern redbelly dace, "*Salvelinus–Semotilus* lakes" containing brook trout and creek chub, and "*Salvelinus–Phoxinus–Semotilus* lakes" containing trout, dace, and chub.

Sport fishermen were given a cooler in which to retain captured brook trout without eviscerating them. Upon the anglers return, length of each individual fish was measured and stomachs were removed and placed in 10% formalin. We assumed that if there was a bias related to this sampling technique, it was the same in all lakes studied. If the stomach content weight to fish weight ratio was lower than 1%, the stomach was considered empty. The different components of the diet were divided into broad functional categories (zooplankton, zoobenthos, terrestrial insects, Hirudinae, and fish) and weighed to ±0.001 g (wet weight). The weight of each category was expressed as a percent of the total stomach contents of a fish. The results were then expressed as the mean percent weight of each prey category (Hyslop 1980). Furthermore, when fish were found in the stomach, the species and number were recorded.

Each study lake was sampled in June 1986, at a time when sportfishing success was at its maximum (sportfishing statistics of the ministère du Loisir, de la Chasse et de la Pêche du Québec), and in July 1986, when benthic resources were presumably at a low level (Mittelbach 1981; Werner 1986; Tremblay and Magnan 1991). Following the results of the first summer, a third sampling was carried out in June 1987 on certain lakes of the *Salvelinus–Phoxinus* and *Salvelinus–Phoxinus–Semotilus* associations.

Laboratory Experiments

Fish and holding conditions

Brook trout, 150–350 mm total length (TL), were captured using experimental multifilament gill nets whereas prey-fish, creek chub (40–170 mm TL) and northern redbelly dace (35–75 mm TL), were captured using minnow traps. Brook trout came from Lake Vison, Mastigouche Reserve (*Salvelinus–Phoxinus–Semotilus* association), the northern redbelly dace from Lake de la Poche, Mastigouche Reserve (*Salvelinus–Phoxinus* association), and the creek chub from Lake Longue-vue, St-Maurice Reserve (*Salvelinus–Semotilus* association). Brook trout and creek chub were acclimatized in rectangular tanks (244 × 60 × 30 cm deep) whereas northern redbelly dace were kept in a circular tank (200 cm in diameter × 40 cm). Photoperiod (12:12), temperature (10 ± 3°C), and physicochemical conditions (pH 7.0 ± 0.2, $NO_2 < 0.2$ mg/L, and $NO_3 < 200$ mg/L) were held constant during the acclimatization period. The photoperiod was maintained with fluorescent lights coupled to a time light system and the water quality using biological filters (East and Magnan 1987). Brook trout were fed with commercial trout pellets (Purina Trout Chow) whereas prey-fish were fed with freeze-dried zooplankton (Nutra Fin). Fish were acclimatized to laboratory conditions for a period of 1 mo.

Experimental apparatus and general protocol

The apparatus consisted of an experimental arena (1.5 × 1.0 × 1 m deep) and four holding chambers equipped with opaque sliding doors in which the predators were placed prior to the beginning of the experiments. During the experiments, photoperiod (12:12), temperature (13 ± 1°C), and physicochemical conditions were kept constant. The water was passed

through a glass wool activated carbon filter between trials. Two weeks prior to the beginning of the experiments, brook trout were conditioned to feed on creek chub and (or) northern redbelly dace of similar size to those used in the experiments. After 5–8 d of conditioning, brook trout learned to associate the opening of the sliding door with the presence of prey-fish in the experimental arena. Experiments on a predator were begun when its predatory behavior (i.e. hunting, capture, and consumption) was initiated rapidly upon presentation of each prey type. For each experiment, the following behaviors were recorded using a DATAMYTE event recorder (model 801): number of attacks, number of captures, amount of time spent hunting and handling prey, and time spent swimming and stationary (on the substrate or in the water column). Brook trout were considered to be in attack phase when swimming became fast, abrupt, and was directed towards a prey, in handling phase from the moment a prey was captured until it was swallowed by the predator, in swimming phase when trout executed natatory movements, initiating and maintaining continuous and directional swimming, and at rest when it was standing stationary in the water column or resting on its pectoral fins on the substrate. The duration of each trial was 10 min, with preys introduced into the experimental arena 10 min before the beginning of the trials. Brook trout were deprived of food 2 d before the beginning of the trials and were randomly assigned to each trial to control for the effect of experience.

Hypothesis testing

To test the first hypothesis, "efficiency of predation on prey-fish increases with the size of brook trout," the three following experiments (i.e. H_2, H_3, and H_4) were carried out on two different brook trout size classes chosen as a result of field observation: 150–250 mm TL (mean ± 1 SD; 232.1 ± 13.4 mm TL) and 250–380 mm TL (289.5 ± 29.4 mm TL), referred to hereafter as <250 and >250 mm. The field study indicated that prey-fish appeared in the diet of trout when individuals reached a length of 200–250 mm (see Results and Discussion section).

To test the second hypothesis, "brook trout is selective with regard to the size of prey-fish," the experiments were conducted with a single prey species, creek chub. This species has a wider range of sizes (40–170 mm TL) than does northern redbelly dace. The prey were divided into three size classes representative of those found in nature; 40–60 mm TL (small), 80–100 mm TL (intermediate), and 130–170 mm TL (large). Five creek chub of each size class were placed in the arena. Four trials were conducted with five small brook trout (<250 mm TL) for a total of 20 trials. Three trials were carried out with each of four large brook trout (>250 mm) for a total of 12 trials.

To test the third hypothesis, "brook trout is selective with regard to prey species," brook trout were exposed to five prey-fish of each species (creek chub and northern redbelly dace). Prey size for each species corresponded to small fish (40–60 mm TL). Five brook trout in each size class (<250 and >250 mm) were used. Five trials were conducted for each predator for a total of 50 trials.

Finally, testing of the fourth hypothesis, "presence of refuges decreased brook trout predation on prey-fish," was aimed at evaluating brook trout predation success on northern redbelly dace in an open environment versus in the presence of refuge. The refuge consisted of a 0.5-m^3 bunch of *Cassandra calyculata* placed in the center of the experimental arena. This type of shelter is the one most commonly found in the littoral zone of the studied lakes (see Naud and Magnan 1988). Ten brook trout, five small individuals (<250 mm) and five large ones (>250 mm), were used for each part of this experiment. Ten dace were used in each trial. Five pairs of trials (i.e. with and without refuge) were conducted with each individual for a total of 100 trials for the two pairs.

Statistical Analyses

For each parameter studied, results among treatments were compared using t tests or analyses of variance (ANOVA), followed by Student–Newman–Keuls multiple-range comparison tests (SNK) (Sokal and Rohlf 1981). Homogeneity of variances was first tested using a F_{max} test (Sokal and Rohlf 1981). When the variances were heterogeneous, the data were log $(x+1)$ transformed. Percentage data (i.e. the proportion of time spent hunting, handling prey, swimming, and at rest) were arcsin $(x^{0.5})$ transformed before analyses (Sokal and Rohlf 1981). For the laboratory experiments, the degrees of freedom were based on the number of fish rather than the number of trials. As we did not identify each fish individually during these laboratory experiments, it was impossible to evaluate the intrafish variance (or calculate a mean value for each fish). We thus assumed that variances computed with trials were representative of those that could have been computed with the mean value for each fish.

Results and Discussion

Field Study

Ontogenetic variations

Within each fish association, the frequency of occurrence of prey-fish found in the stomach contents of brook trout increased with trout body size (Fig. 1). This relationship has been previously documented in the field and laboratory (Campbell 1979; Garman and Nielsen 1982; Tonn and Paszkowski 1986), and it suggests a reason for the low predation success of brook trout in our study system; only 21% of brook trout in our samples were >300 mm TL, and it is especially from this size that predation success was highest (Fig. 1). The low proportion of large individuals is due to the exploitation of these populations through sportfishing, which tends to reduce the mean size of trout.

Intercommunity variations

The mean length of brook trout in the *Salvelinus–Phoxinus* association was significantly higher than that of brook trout in the *Salvelinus–Phoxinus–Semotilus* association, which in turn was significantly higher than that of brook trout in the *Salvelinus–Semotilus* association ($F = 95.6$, $p < 0.001$; Table 1). We observed that brook trout consumption of prey-fish was not frequent in the *Salvelinus–Semotilus* association (2 of the 10 cases studied, i.e. 20%, number of lakes × number of sampling periods). In contrast, predation on fish was more frequent in the *Salvelinus–Phoxinus* association (10 of the 22 cases studied, i.e. 46%) and in the *Salvelinus–Phoxinus–Semotilus* association (10 of the 14 cases studied, i.e. 71%). The mean percent weight of fish found in brook trout stomachs was significantly higher in the *Salvelinus–Phoxinus–Semotilus* association (7.2 ± 9.7%) than in *Salvelinus–Phoxinus* (3.4 ± 5.7%) and *Salvelinus–Semotilus* (0.9 ± 2.7%) associations ($F = 3.17$, $p < 0.05$; Table 2). We observed that prey-fish found in brook

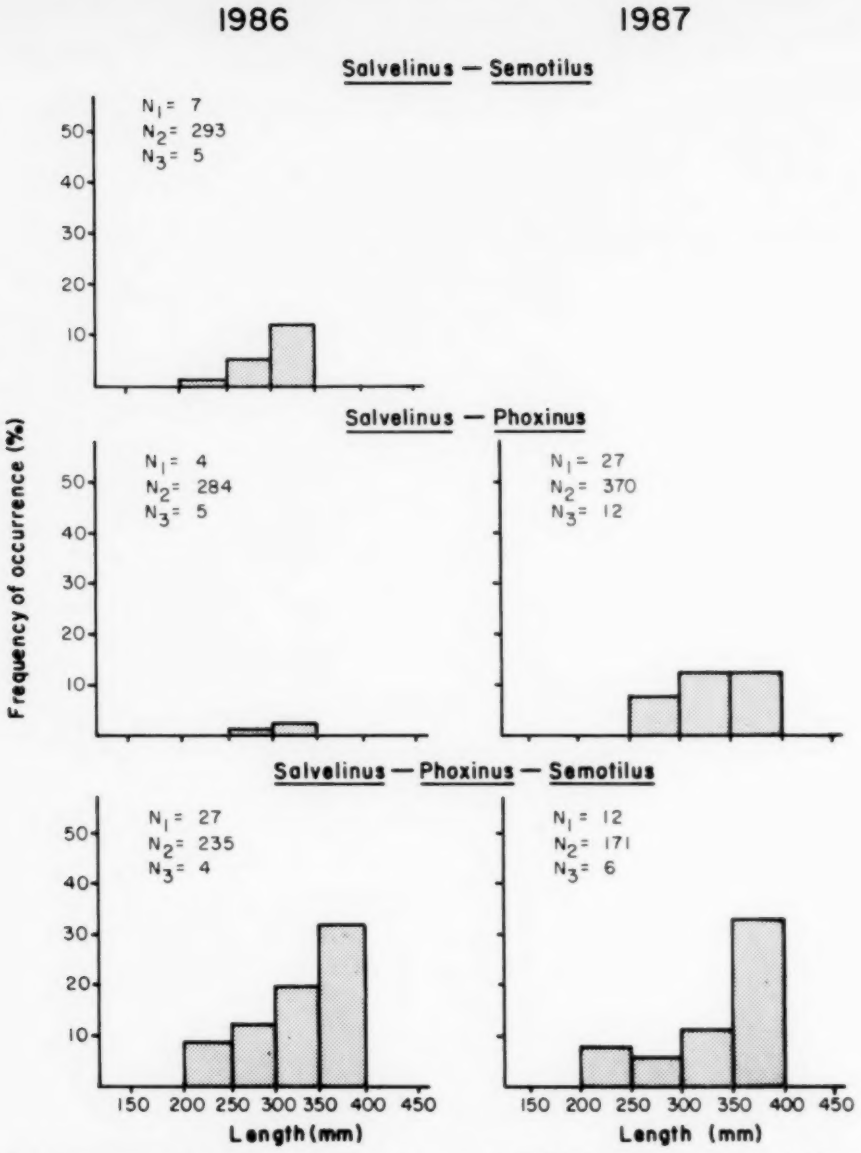

FIG. 1. Occurrence of prey-fish in the stomach contents of brook trout according to size in the three fish associations studied in 1986 and 1987. N_1, occurrence of fish in the stomach content of trout; N_2, number of stomach contents analyzed; and N_3, number of study lakes.

trout stomachs were almost exclusively northern redbelly dace, even in the *Salvelinus–Phoxinus–Semotilus* association (only 2 of the 39 prey-fish found in the stomach contents of trout from this association were creek chub). These results on fish predation indicate that brook trout seldom feed on or select creek chub as prey-fish. The low predation on creek chub could be explained by the smaller size of trout in this fish association and (or) the spatial distribution of the individuals. Magnan and FitzGerald (1984) mentioned that small creek chub (i.e. <80 mm), the most likely to be preyed on (see laboratory experiments below), were found almost exclusively within 0.8 m depth along the shore. Northern redbelly dace is presumed to be more vulnerable than creek chub to predation because of its onshore–offshore diel feeding migrations (Naud and Magnan 1988). Second, the results suggest that predation on northern redbelly dace is mediated by the presence of creek chub because predation on dace was significantly higher (mean percent weight) and more frequent in *Salvelinus–Phoxinus–Semotilus* lakes than in *Salvelinus–Phoxinus* lakes (Table 2; Fig. 1), even though trout were significantly smaller in the *Salvelinus–Phoxinus–Semotilus* than in the *Salvelinus–Phoxinus* association (Table 1). Such an indirect effect of one species on the interactions between two other species was observed in a community of bream, *Abramis brama*, eel, *Anguilla anguilla*, and smelt, *Osmerus eperlanus* (Lammens et al. 1985).

TABLE 2. Mean percent weight of each prey and mean weight of stomach contents (± 1 SD) of brook trout found in the different fish associations of the three sampling dates.

Sampling date	No. of lakes	Mean percent weight of prey						
		Plankton	Benthos	Terrestrial insects	Hirudinae	Other	Fish	Mean weight (g)
				Salvelinus – Phoxinus				
June 1986	5	24.3 ± 16.1	59.9 ± 18.6	7.7 ± 9.8	1.0 ± 1.0	5.9 ± 7.0	1.1 ± 1.6	1.99 ± 2.46
July 1986	5	21.1 ± 7.9	71.4 ± 13.1	4.6 ± 6.1	1.6 ± 2.2	0.3 ± 0.5	0.9 ± 2.1	1.11 ± 1.36
June 1987	12	29.2 ± 26.2	60.2 ± 23.9	2.8 ± 4.3	0.3 ± 0.6	2.1 ± 1.5	5.4 ± 7.1	1.19 ± 1.31
Total	22	26.2 ± 20.8	62.7 ± 20.5	4.3 ± 6.3	0.7 ± 1.2	2.6 ± 3.8	3.4 ± 5.7	1.36 ± 1.69
				Salvelinus – Semotilus				
June 1986	5	28.0 ± 26.6	64.0 ± 26.4	4.4 ± 5.6	0.2 ± 0.5	1.6 ± 1.0	1.8 ± 3.8	1.07 ± 1.26
July 1986	5	31.9 ± 27.7	59.1 ± 28.7	7.5 ± 4.8	0.1 ± 0.3	1.6 ± 1.4	0.0 ± 0.0	0.67 ± 0.82
Total	10	29.9 ± 25.7	61.6 ± 26.1	5.9 ± 5.2	0.2 ± 0.4	1.6 ± 1.2	0.9 ± 2.7	0.88 ± 1.09
				Salvelinus – Phoxinus – Semotilus				
June 1986	4	44.3 ± 25.2	36.8 ± 20.9	8.5 ± 4.9	3.9 ± 4.1	2.5 ± 2.9	6.9 ± 5.4	1.25 ± 1.42
July 1986	4	39.3 ± 36.1	44.3 ± 31.4	6.4 ± 5.2	1.7 ± 2.5	0.1 ± 0.1	8.2 ± 10.7	1.18 ± 2.42
June 1987	6	45.1 ± 26.3	41.8 ± 23.8	3.0 ± 5.1	1.1 ± 2.3	2.1 ± 2.6	6.8 ± 2.5	0.76 ± 1.31
Total	14	43.2 ± 26.8	41.1 ± 23.6	5.6 ± 5.3	2.1 ± 3.0	1.3 ± 2.2	7.2 ± 9.7	1.16 ± 1.42

TABLE 3. Mean percent of fish by weight in the stomach contents of brook trout of the *Salvelinus–Phoxinus–Semotilus* association on the three sampling dates (number of stomachs analyzed in parentheses).

Lake	June 1986	July 1986	June 1987	Total
Cutaway	6.3 (26)	0.0 (25)	0.0 (25)	2.1 (76)
Du Camp	8.4 (25)	23.2 (26)	4.6 (26)	12.1 (77)
French	12.9 (26)	8.6 (25)	32.1 (22)	17.2 (73)
Vison	0.0 (27)	1.0 (29)	3.3 (27)	1.4 (83)
Bourassa	—[a]	—[a]	0.7 (27)	0.7 (27)
Argent	—[a]	—[a]	0.0 (5)	0.0 (5)

[a]Not sampled in 1986.

Intracommunity variations

We observed that the feeding behavior of brook trout varied widely from lake to lake. In the *Salvelinus–Phoxinus–Semotilus* association, the degree of piscivory by brook trout was high in two lakes (Du Camp and French) but was much lower in the other four lakes (Table 3). These results suggest that certain habitat parameters, specific to these lakes, might determine brook trout piscivorous behavior. Speirs (1974) noted a relationship between the surface area of a lake and the predation rate for brook trout. Lakes with a large offshore zone could maintain larger populations of zooplanktivorous prey-fish; in those cases, brook trout became piscivorous. In contrast, we found an inverse correlation between the surface area of the lakes and the mean percent weight of fish in the stomachs of trout in the *Salvelinus–Phoxinus–Semotilus* association ($n = 6$, $r = -0.86$, $p < 0.05$). Lakes French and Du Camp had in common a higher structural complexity in the littoral zone (i.e. floating vegetative cover and submerged trees resulting from past logging; P. East, pers. obs.). S. Lacasse and P. Magnan (unpubl. data) found that values of an index of structural complexity for Lake Du Camp were among the highest in a group of 38 study lakes of the same area but found no relationship between lake area and structural complexity of the littoral zone. It is possible that higher structural complexity in certain lakes allows brook trout to adopt a "sit-and-wait forager" strategy (individuals that move only to intercept a prey and then return to a stationary position) instead of an "active forager" strategy (individuals that swim slowly between forages; both as defined by Grant and Noakes 1987) and thus increase their predation success on prey-fish (see also Results and Discussion of laboratory experiments).

Laboratory Experiments

Effect of predator and prey sizes

Small brook trout (<250 mm) attacked small creek chub (40–60 mm) significantly more often than they did intermediate (80–100 mm) and large ones (130–170 mm) ($F = 15.14$, $p < 0.05$; Table 4). Furthermore, only small creek chub were captured by small brook trout (Table 4). In contrast, we did not observe any significant difference in the number of attacks ($F = 2.00$, $p = 0.15$) or in the number of captures ($F = 1.46$, $p = 0.24$) on the three size classes of prey-fish for large brook trout (>250 mm; Table 4). However, large chub apparently needed a longer handling time than intermediate and small ones (Table 4). Werner (1974), Bannon and Ringler (1986), and Hoyle and Keast (1987) determined that for smallmouth bass, *Micropterus dolomieui*, and largemouth bass, *Micropterus salmoides*, the handling time increased abruptly with prey size. During our experiments, small brook trout spent a significantly greater time at rest (in the water column or on the substrate) than large individuals (56.6 ± 23.0% versus 32.0 ± 26.4%; $t = 2.78$, $p < 0.05$) while spending comparable periods of time swimming (31.9 ± 21.2%; $t = 1.05$, $p = 0.31$), attacking (14.3 ± 15.6%; $t = 1.11$, $p = 0.29$), and handling prey (16.4 ± 20.9%; $t = 0.99$, $p = 0.35$).

Effect of the prey species available

We did not observe any significant difference in the handling time of the two prey species available, although both size classes

TABLE 4. Predation of brook trout with regard to fish size (number of attacks, number of captures, and handling time). Data are means ± 1 SD (number of trout used in each size class in parentheses).

	Salvelinus < 250 mm (n = 5)			*Salvelinus* > 250 mm (n = 4)		
	Semotilus (40–60 mm)	*Semotilus* (80–100 mm)	*Semotilus* (130–170 mm)	*Semotilus* (40–60 mm)	*Semotilus* (80–100 mm)	*Semotilus* (130–170 mm)
No. of trials		20			12	
No. of attacks per 10 min	13.85 ± 11.68 *a*	5.30 ± 4.61 *b*	1.60 ± 2.50 *b*	9.08 ± 8.48 *a*	6.00 ± 6.81 *a*	4.92 ± 4.79 *a*
No. of captures per 10 min	0.64 ± 0.79	—	—	0.42 ± 0.67 *a*	0.17 ± 0.39 *a*	0.25 ± 0.45 *a*
Handling time (min)						
First prey	0.541 ± 0.458 (8)[b]	—	—	0.115 ± 0.064 (4)[a]	1.125 ± 0.714 (2)[a]	4.803 ± 3.004 (3)[a]
Second prey	2.320 ± 0.021 (2)	—	—	0.170 (1)	—	—
Total[c]	0.898 ± 0.853 (10)	—	—	0.126 ± 0.064 (5)[a]	1.125 ± 0.714 (2)[a]	4.803 ± 3.004 (3)[a]

NOTE: In each experiment (predator size), means followed by the same italicized letter are not significantly different as determined by an ANOVA followed by a Student-Newman-Keuls multiple-range comparison test (F and p values are given in the text).

[a]Indicates that the sample was too low to allow statistical analyses.

[b]Sample size.

[c]Mean handling time (min) of total number of prey captured by a predator in one experiment.

TABLE 5. Predation of brook trout with regard to fish species (number of attacks, number of captures, and handling time). Data are means ± 1 SD (number of trout used in each size class in parentheses).

	Salvelinus <250 mm (n = 5)		*Salvelinus* >250 mm (n = 5)	
	Phoxinus	*Semotilus*	*Phoxinus*	*Semotilus*
No. of trials	25	25	25	25
No. of attacks per 10 min	9.30 ± 4.18 *a*	4.91 ± 3.05 *b*	10.76 ± 4.79 *a*	7.36 ± 4.49 *b*
No. of captures per 10 min	1.61 ± 0.78 *a*	0.26 ± 0.45 *b*	1.76 ± 1.05 *a*	0.64 ± 0.70 *b*
Handling times (min)				
First prey	0.103 ± 0.075 (24) *a*[b]	0.193 ± 0.170 (6) *a*	0.070 ± 0.053 (22) *a*	0.148 ± 0.106 (13) *a*
Second prey	0.431 ± 0.403 (13)[a]	—	0.289 ± 0.356 (15)[a]	1.153 ± 0.705 (3)[a]
Total[c]	0.237 ± 0.316 (40) *a*	0.193 ± 0.170 (6) *a*	0.182 ± 0.244 (44) *a*	0.336 ± 0.489 (16) *a*

NOTE: In each experiment (predator size), means followed by the same italicized letter are not significantly different as determined by a t test (t and p values are given in the text).

[a]Indicates that the samples was too low to allow statistical analyses.

[b]Sample size.

[c]Mean handling time (min) of total number of prey captured by a predator in one experiment; note that more than two prey were often captured by a trout.

of brook trout tended to have lower handling time for northern redbelly dace than for creek chub (<250 mm: $t = 0.58$, $p = 0.57$; >250 mm: $t = 1.21$, $p = 0.24$; Table 5). The two size classes of trout showed a significant preference for northern redbelly dace with regard to the number of attacks (<250 mm: $t = 4.76$, $p < 0.05$; >250 mm: $t = 3.17$, $p < 0.05$; Table 5) and a significantly greater efficiency in terms of number of captures (<250 mm: $t = 10.19$, $p < 0.01$; >250 mm: $t = 6.78$, $p < 0.01$; Table 5). These results indicate that northern redbelly dace is both selected more frequently (number of attacks) and is more vulnerable (number of captures) than creek chub, regardless of predator size. These results agree with those obtained in nature where almost all fish ingested were northern redbelly dace. The greater vulnerability of northern redbelly dace, compared with creek chub of comparable size, is possibly due to their slower reaction time when faced with a predator (P. East, pers. obs.) and also to the ease with which they can be handled. We did not observe any significant difference in the average time spent at rest (in the water column or on the substrate) (65.7 ± 22.3%; $t = 0.33$, $p = 0.74$), swimming (21.7 ± 18.5%; $t = 0.55$, $p = 0.58$), attacking (7.9 ± 6.4%; $t = 2.68$, $p = 0.07$) and handling prey (5.3 ± 5.4%; $t = 0.29$, $p = 0.78$) between small and large brook trout.

Effect of refuge for prey fish

The number of attacks and captures was significantly lower in presence of a refuge, whatever the predator size (number of attacks — <250 mm: $t = 5.15$, $p < 0.05$; >250 mm: $t = 6.85$, $p < 0.01$; number of captures — <250 mm: $t = 5.09$, $p < 0.05$; >250 mm: $t = 6.68$, $p < 0.01$; Table 6). Savino and Stein (1982) found reduced predation success by largemouth bass on bluegills, *Lepomis macrochirus*, in the presence of high densities of simulated aquatic vegetation. We observed that as soon as the prey fish were placed in the experimental arena, all individuals took refuge among *Cassandra* stems, even in the absence of a predator. Fraser (1983) observed the same type of behavior in creek chub; individuals actively looked for a shelter to counter an eventual attack from brook trout.

TABLE 6. Effect of the presence of refuges (*Cassandra calyculata*) on brook trout predation (number of attacks, number of captures, time swimming, time at rest, time for attacks, and handling time). Data are means ± 1 SD (number of trout used in each size class in parentheses).

	Salvelinus <250 mm (n = 5)		*Salvelinus* >250 mm (n = 5)	
	With refuges	Without refuges	With refuges	Without refuges
No. of trials	25	25	25	25
No. of attacks per 10 min	6.80 ± 4.10 *a*	20.32 ± 12.46 *b*	7.92 ± 5.82 *a*	25.12 ± 11.12 *b*
No. of captures per 10 min	0.56 ± 0.71 *a*	2.32 ± 1.31 *b*	0.68 ± 0.64 *a*	3.68 ± 2.13 *b*
Time swimming (min)	2.74 ± 1.57 *a*	2.90 ± 1.52 *a*	2.60 ± 0.98 *a*	3.63 ± 1.88 *b*
Time at rest (min)	6.94 ± 1.59 *a*	5.86 ± 2.08 *a*	7.09 ± 1.14 *a*	5.02 ± 2.03 *b*
Time for attacks	0.57 ± 0.65 *a*	1.35 ± 0.80 *b*	0.43 ± 0.19 *a*	1.45 ± 0.64 *b*
Handling time (min)				
First prey	0.121 ± 0.083 (12) *a*[b]	0.093 ± 0.074 (24) *a*	0.054 ± 0.042 (14) *a*	0.057 ± 0.050 (25) *a*
Second prey	0.440 (1)[a]	0.383 ± 0.491 (17)[a]	0.106 ± 0.042 (3)[a]	0.155 ± 0.197 (21)[a]
Total[c]	0.205 ± 0.248 (14) *a*	0.302 ± 0.379 (58) *a*	0.063 ± 0.045 (17) *a*	0.139 ± 0.150 (92) *b*

NOTE: In each experiment (predator size), means followed by the same italicized letter are not significantly different as determined by a t test (t and p values are given in the text).

[a]Indicates that the sample was too low to allow statistical analyses.

[b]Sample size.

[c]Mean handling time (min) of total number of prey captured by a predator in one experiment; note that more than two prey were often captured by a trout.

There was no significant difference in the handling time of the first prey (<250 mm: $t = 1.00$, $p = 0.33$; >250 mm: $t = 0.19$, $p = 0.85$) and the second prey (<250 mm: $t = 0.48$, $p = 0.64$; >250 mm: $t = 1.01$, $p = 0.32$) captured, with or without cover (Table 6). When cover was available, brook trout of both size classes never captured more than two prey during the 10 min of a trial. However, in the absence of cover, brook trout captured generally twice as many prey, and increased the handling time at each additional prey (Table 6). With regard to time allocated to each behavior, we observed that large brook trout spent significantly more time at rest (in the water column or on the substrate) when there was cover available ($t = 4.46$, $p < 0.05$; Table 6). However, small brook trout did not show any significant difference in the time spent at rest with or without available refuge ($t = 2.06$, $p > 0.05$; Table 6). The period of time allocated to attack was significantly reduced for brook trout of both size classes in the presence of cover (<250 mm: $t = 3.82$, $p < 0.05$; >250 mm: $t = 12.49$, $p < 0.001$; Table 6). The result of these two changes in behavior among large brook trout means that they adopted a "sit-and-wait forager" behavior (Grant and Noakes 1987); individuals spent significantly more time at rest and carried out only brief attacks on fish which occasionally left the cover. In the absence of cover, brook trout opted for an "active forager" behavior (Grant and Noakes 1987), swimming almost continually and carrying out attacks that broke the cohesion of prey-fish schools. Although statistical analyses did not allow us to confirm this shift of behavior in small brook trout, laboratory observations indicated a similar trend (P. East, pers. obs.).

Conclusions

Stomach content analysis as well as laboratory experiments suggested that fish such as northern redbelly dace can form an important part (i.e. up to 30% by weight) of the diet of brook trout. However, these results also suggest that piscivory is regulated by certain biotic and abiotic factors. We observed, in both the field and laboratory, that predation success increased with predator size. Brook trout started to eat fish after reaching 200 mm, and the amount of fish consumed increased rapidly for individuals greater than 300 mm. A second biotic factor that seems involved in brook trout predation success is the fish community structure. We observed that frequency of predation on dace was significantly higher in the *Salvelinus–Phoxinus–Semotilus* association than in the *Salvelinus–Phoxinus* association. This may be explained by the niche shift of brook trout from zoobenthos to zooplankton when they live in sympatry with creek chub (Magnan and FitzGerald 1982; Magnan 1988). Fish may be a more profitable prey for sympatric brook trout than zoobenthos. Engen et al. (1988) provided theoretical and empirical evidence that ranking of food items may change in the presence of competition.

One abiotic factor clearly involved in brook trout predation success is the presence of refuges for prey-fish. The laboratory experiments showed that when cover was available, brook trout predation success was reduced. Thus, it would be advantageous for northern redbelly dace to use this type of refuge to reduce the risk of predation from brook trout. In a recent study, Naud and Magnan (1988) showed that northern redbelly dace used such refuges in their natural habitat. They observed that northern redbelly dace swam in shoals in the littoral zone during the day, migrated to the offshore zone at sunset where the shoals break up into single fish, and then returned to the littoral zone at sunrise. The results of the latter study indicated that the diel migrations to the offshore zone at nightime allowed individuals to increase their feeding efficiency on zooplankton while reducing the risk of predation from brook trout, a visually oriented predator. Furthermore, northern redbelly dace showed a significant preference for habitat with dense cover (*Cassandra calyculata* type) compared with habitat with sparse cover (*Sparganium* sp.) in the littoral zone during the day (Naud and Magnan 1988). This result combined with our laboratory exper-

iments suggests that this behavior protects them from brook trout predation.

Other abiotic factors may also be involved in brook trout predation success on prey-fish. We observed that the degree of piscivory varied widely from lake to lake in *Salvelinus–Phoxinus–Semotilus* lakes. Certain habitat factors related to the structural complexity of the littoral zone, such as floating vegetative cover and submerged trees, could determine the degree of brook trout piscivorous behavior.

Brook trout rarely preyed on creek chub, and predation on northern redbelly dace was low as a whole in the lakes and fish communities we examined. Our experiments showed that trout of both size classes significantly preferred northern redbelly dace. Predation on northern redbelly dace was low in the field because most brook trout sampled were smaller than 250 mm in length, and laboratory experiments clearly indicated that trout of these sizes were less effective in preying on dace than larger ones. Predation on northern redbelly dace would probably be higher in lakes with larger predators.

Acknowledgements

We thank S. Lachance and M. Proulx for their invaluable field assistance and J. Archambault and C. Garceau of the ministère du Loisir, de la Chasse et de la Pêche du Québec, for logistic support. We are also grateful to Drs. G. Houle, F. G. Whoriskey, W. M. Tonn, and one anonymous referee for their comments on the manuscript. Financial support was provided by the Natural Sciences and Engineering Research Council of Canada and the ministère du Loisir, de la Chasse et de la Pêche du Québec, to P. Magnan. P. East was supported by a postgraduate fellowship from le Fonds pour la formation de chercheurs et l'aide à la recherche, Gouvernement du Québec.

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Two Generations of Hybrids between Even- and Odd-Year Pink Salmon (*Oncorhynchus gorbuscha*): A Test for Outbreeding Depression?

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Gharrett, A. J., and W. W. Smoker. 1991. Two generations of hybrids between even- and odd-year pink salmon (*Oncorhynchus gorbuscha*): a test for outbreeding depression? Can. J. Fish. Aquat. Sci. 48: 1744–1749.

Hybrids of genetically isolated odd- and even-year pink salmon (*Oncorhynchus gorbuscha*) from the same stream were made by fertilizing eggs with cryopreserved milt. Anadromous first-generation (F_1) hybrids and controls returned to the hatchery at equal rates (153 of 5483 and 160 of 5492, respectively), on the same average date, and with the same size. However, variances of F_1 size (female length and weight and male length) exceeded variances of control sizes, suggesting increased genetic variation in F_1's. Only 11 of 5165 F_2's returned. F_2's were similar meristically and in size to fish of their parents' generation, but were bilaterally more asymmetric in number of gill rakers and in combined numbers of gill rakers and of branchiostegals. Increased F_1 variation followed by low F_2 returns and increased bilateral asymmetry is a pattern to be expected when coadapted allele complexes are disrupted by outbreeding depression.

Nous avons créé des hybrides de saumons roses (*Oncorhynchus gorbuscha*) d'années paires et impaires, génétiquement isolés, provenant du même cours d'eau, en fécondant des oeufs avec du sperme cryoconservé. Les hybrides anadromes de première génération (F_1) et les témoins sont revenus à l'écloserie dans les mêmes proportions (respectivement 153 sur 5 483 et 160 sur 5 492), à la même date moyenne et à la même taille. Les variances de la taille des F_1 (longueur et poids des femelles et longueur des mâles) dépassaient toutefois les variances de la taille chez les témoins, ce qui indiquerait un accroissement de la variation génétique chez les F_1. Onze seulement des 5 165 F_2 sont revenus. Les F_2 étaient similaires sur le plan méristique et en taille aux poissons de la génération de leurs parents, mais présentaient davantage d'asymétrie bilatérale en ce qui concerne le nombre de branchicténies et le nombre combiné de branchicténies et de rayons branchiostèges. Une augmentation de la variation chez les F_1 suivie par de faibles retours des F_2 et une hausse de l'asymétrie bilatérale constituent donc le scénario à prévoir lorsqu'on perturbe des complexes d'allèles coadaptés par une dépression due au croisement.

Received October 29, 1990
Accepted March 13, 1991
(JA775)

Reçu le 29 octobre 1990
Accepté le 13 mars 1991

The effects of hatchery and farmed fish on wild stocks of salmonids continue to be a contentious issue (e.g. Baranaga 1990; Pitts 1990). One concern is the genetic impact on local native populations by hatchery stocks derived from transplants from remote populations (Helle 1981; Krueger et al 1981). A component of this concern is the potential for outbreeding depression (Shields 1982). The term "outbreeding depression" denotes the decrease in average survival or fitness which results from hybridizing individuals from different populations and may result because fish from the two populations have different genomes. Outbreeding depression is more severe when dissimilar parents are hybridized, for example parents from widely separated populations (Shields 1982).

One explanation for outbreeding depression is that reproductively isolated native populations may have evolved unique genetic complements comprising coadapted, interacting (epistatic) alleles at a large number of loci (Wallace 1981). The coadapted set of alleles of the native population is put at risk by introgression of alleles from imported hatchery fish which, most likely, possess an alternative coadapted set derived from an independent coadaptation process. The coadapted sets of alleles can differ if each population was founded with different complements of genetic material and/or each underwent independent selection regimes. Outbreeding depression from interpopulational crosses may be preceded by heterosis or luxuriance in the first generation (F_1). However, decreased fitness (e.g. reductions in fitness-related traits such as longevity and fecundity) from a breakdown of coadapted sets of alleles follows in the second (F_2) and subsequent generations (e.g. Dobzhansky 1948, 1950; Brncic 1954; Wallace and Vetukhiv 1955). In some cases the breakdown is actually observed in the F_1 generation (King 1955). The majority of research on coadaptation of alleles in populations has been on *Drosophila*; however, outbreeding depression has been reported for a variety of

other species (Wallace 1981; Shields 1982) including fish (Gordon and Gordon 1957). Moreover, distinct subpopulations with unique, coadapted allele complements can occur even in the presence of gene flow (Endler 1977).

There is reason to expect that interpopulational crosses of anadromous Pacific salmon may exhibit outbreeding depression. The fidelity of homing by anadromous Pacific salmon enforces reproductive isolation resulting in genetic differentiation which is often reflected by differences in allozyme frequencies among populations or other hierarchical levels of population structure. Evidence from pink salmon populations including those studied here was found by McGregor (1982) and Gharrett et al. (1988). Such isolated units may also evolve distinct, coadapted allele complexes. In addition, there is some evidence that experimental outbreeding results in diminished homing ability, a behavioral barrier to outbreeding in nature, in F_1 pink salmon (*Oncorhynchus gorbuscha*) (Bams 1976). In contrast, one would not expect to see outbreeding depression appear following hybridization of populations between which substantial gene flow (straying) occurred. For pink salmon, this would probably apply to populations that share temporal and geographical similarities.

To examine the hypothesis that outbreeding depression can result from interpopulational crosses of genetically isolated salmon populations, we have produced and examined F_1 and F_2 pink salmon from hybrids of even-year males and odd-year females from the same stream. Pink salmon are uniquely appropriate to test this hypothesis because, in their natural range, they have a rigid 2-yr, semelparous life cycle to which exceptions are exceedingly rare (Gilbert 1913; Davidson 1934; Rounsefell 1958; Anas 1959; Bilton and Ricker 1965; Turner and Bilton 1968). The temporal separation resulting from the life cycle of this species has produced in many streams and rivers two genetically distinct lines (Aspinwall 1974; Johnson 1979; McGregor 1982; Beacham et al. 1988) which spend their freshwater life in the same stream.

Another intrinsic objective of this work was to recover and evaluate returns of F_1 and F_2 hybrids between even- and odd-year pink salmon. In the only previous experiment that released even- by odd-year pink salmon hybrids, there were no F_1 returns to the hatchery and no opportunity to produce F_2 hybrids (Withler and Morley 1982).

Materials and Methods

Auke Creek is located in northern southeastern Alaska 18 km from Juneau. It flows about 350 m from Auke Lake into Auke Bay. The U.S. National Marine Fisheries Service Auke Bay Laboratory operates a weir and salmon hatchery at the mouth of the creek at the head of tidewater and obtains precise counts of both emigrant fry and returning adults. Natural runs of both even- and odd-year pink salmon return to the steam. Within each year class, there are five identifiable subpopulations: early-run intertidal and early-run upstream subpopulations which enter Auke Creek and spawn in Late July and August, late-run intertidal and late-run upstream subpopulations which enter and spawn in September, and a subpopulation which spawns in Lake Creek, a tributary to Auke Lake. Returning adults marked by fin clips (Taylor 1980) and a genetic marker (Lane et al. 1990) have demonstrated that subpopulations of pink salmon within Auke Creek home accurately with respect to both time and space.

On 25 September 1982 we cryopreserved sperm from each of 20 late-run upstream males by pelleting it on solid CO_2 after diluting it (1:3) in an ice-cold glucose–DMSO extender (300 mM glucose : DMSO, 10:1) (Stoss and Refstie 1983). We stored the pellets from each male in separate containers in or over liquid N_2 until use in fall 1983.

We produced controls and hybrids between brood years on 17 September 1983. Ova from 20 late-run upstream females (some of which were partially spent) were pooled, gently mixed, and divided into two lots. We fertilized one lot with the thawed sperm of 1982 males to produce hybrids. From the other lot, we produced a control group which was fertilized with never-frozen, fresh sperm from 20 late-run upstream males that returned in 1983. We used two methods to thaw cryopreserved sperm and make between-year hybrid crosses. The first hybrids were made in small lots by thawing 10 pellets in 10 mL of 120 mM $NaHCO_3$ at 25–30°C, agitating for several seconds, and dry-fertilizing approximately 100 ova (Stoss and Refstie 1983). Because this method proved time consuming, we expanded the scale approximately 10-fold and used combinations of pellets from several different males to fertilize about 1000 ova at a time. The twenty 1982 males contributed roughly equally to the F_1 generation.

F_1 individuals returning in 1985 were bred to produce a second generation of interyear hybrids. On 10, 14, 15, and 17 September we combined pooled gametes of one female and two males, of five females and eight males, of seven females and eight males, and of 16 females and 14 males, respectively, to produce an F_2 generation which had a maximum effective population size (N_e) of 59.8 fish (Gharrett and Shirley 1985).

Both F_1 and F_2 experiments were cultured and marked using the same procedure. Fertilized eggs were incubated in Heath-Tecna™ incubators until eyed, dead eggs were discarded, and development was completed in gravel incubators (Bailey and Taylor 1974). Mortality rates were not quantified. All hybrid fry were marked by clipping the adipose and left pelvic fins and controls by clipping the adipose and right pelvic fins. Samples of fry clipped by each worker each day demonstrated the high quality (>95% properly marked) of the process. No returns in 1987 had a single clip (either adipose only or pelvic only), which also indicates high quality of the marking process and low rate of fin regeneration. On 6 April 1984, 5483 marked F_1 hybrid and 5492 marked control pink salmon were released from Auke Creek Hatchery. On 5 April 1986, 5156 marked F_2 hybrids were released. No marked controls were released for the F_2's.

Returning marked experimental fish were recovered at the weir and held in pens until they were processed; none of them were allowed to spawn in Auke Creek. Sex, date of return, weight, and length (mideye to fork of tail) of returning fish were recorded. A uniquely numbered plastic disc was affixed to each fish and the fish were frozen at −20°C until we could count left and right branchiostegals, gill rakers, and pectoral fin rays.

We compared relative rates of return and means or variances of characteristics observed in hybrid and control groups. Because the distributions of measurements on females differ from the distributions observed for males, we analyzed the male and female data independently. Equality of means was tested assuming that variances of hybrid and control groups were unequal (Winer 1971, p. 41–43). Because *F*-tests are quite sensitive to departures from normality, they are often inappropriate for testing equality of variances (Van Valen 1978). Therefore, we performed one-tailed *t*-tests between variances using Lev-

TABLE 1. Returns for between-year F_1 pink salmon hybrids and odd-year controls returning in 1985 and for F_2 hybrids returning in 1987. No controls were released for brood year 1985; percent returns to Auke Creek are presented for late-run naturally spawned fish. Numbers of males (m) and females (f) returning are in parentheses. Data for returns from brood year 1983 of seven additional fish (six males and one female) are missing.

Release group	Return year	Number emigrating	Number returned	Percent return
Even–odd hybrids	1985	5 483	153 (81 m, 72 f)	2.79
Odd-year controls	1985	5 492	160 (91 m, 69 f)	2.91
Late upstream wild fish	1985	95 749[a]	5040[a]	5.26
F_2 hybrids	1987	5 165	11 (8 m, 3 f)	0.21
Late upstream wild fish	1987	59 666[a]	2430[a]	4.07

[a]Values estimated using daily counts of emigrant fry and frequencies of a genetic marker bred into the late run (Lane et al. 1990).

TABLE 2. Average date of return in September, length, and weight of controls (all parents mature in 1983), F_1 hybrids (dams nature in 1983 and sires in 1982), and F_2 hybrids (offspring of F_1 hybrids). Superscripts on means of F_1 hybrids denote probability of equality of F_1 and control means (two-tailed tests), superscripts on F_1 variances denote probability that they exceed control variances (one-tailed tests), and superscripts on F_2 means denote the probability of equality of means of F_1 and F_2 hybrids ([a]$P>0.10$; [b]$P<0.10$; [c]$P<0.05$; [d]$P<0.001$).

	Females			Males		
	Control	F_1	F_2	Control	F_1	F_2
			Return date (September)			
Mean	15.71	15.78[a]	4.00[b]	14.42	14.65[a]	2.75[d]
n	69	72	3	91	81	8
Variance	20.80	19.25[a]	27.00	31.93	28.65[a]	5.64
			Length (mm)			
Mean	493.71	496.24[a]	475.00[b]	480.00	473.74[a]	471.88[a]
n	68	72	3	90	81	8
Variance	326.00	502.72[c]	225.00	845.73	1229.52[b]	220.98
			Weight (kg)			
Mean	1.567	1.572[a]	1.380[a]	1.496	1.421[a]	1.458[a]
n	61	67	3	86	71	8
Variance	0.044	0.069[b]	0.020	0.094	0.114[a]	0.418

ene's test (Millikan and Johnson 1984, p. 19–23) to test if variances of hybrids exceeded variances of controls. The *t*-tests using jackknife estimates of the variances of the variances (Arvesen and Schmitz 1979) produced comparable results.

We used log-likelihood ratios (*G*-tests, Sokal and Rohlf 1981) or exact binomial probabilities to compare return rates and proportions of fish that had asymmetrical counts.

Results

Between 23 August and 24 September 1985, 153 F_1 hybrid and 160 control fish returned to the Auke Creek weir. The majority returned after 7 September and were clearly part of the late run (Taylor 1980; Lane et al. 1990) from which they were derived. Survivals of the two groups, as estimated by return rates, were virtually identical ($P = 0.70$). These rates were somewhat lower than that estimated for unmarked late-run fish which spawned naturally and did not have a marking handicap (Table 1).

Averages and variances of the time (date) of return did not differ between F_1 and control groups. However, variances of weights and lengths were larger for F_1 hybrids, and three of four were significantly larger ($P < 0.10$) (Table 2).

Counts of gill rakers and branchiostegals were similar for F_1 and control females, but F_1 males averaged approximately one half gill raker more than control fish on both the left and right sides (Table 3). This difference was significant (left, $P < 0.05$; right, $P < 0.01$) because the variances observed for the meristic counts were quite small. The male F_1 hybrids also differed in numbers of left and right branchiostegals ($P < 0.05$). A difference in extent of asymmetry in bilateral counts of branchiostegals ($P < 0.10$) was observed between F_1 and control females (Table 4). Only small (insignificant) differences between hybrids and controls were observed among pectoral fin ray counts; those data are not presented.

Between 1 and 10 September 1987, three female and eight male F_2 hybrids returned to the Auke Creek weir (Table 1). The return rate was 0.21%, less than 1/10 of the return rate for the F_1's and controls in 1985 and much lower than that estimated for the late-run naturally produced pink salmon returning to Auke Creek in 1987. Although the average time (date) of return was significantly earlier ($P < 0.10$ for females and $P << 0.001$ for males) than was observed for the 1985 return, they were similar to those of the late runs in the area and in Auke Creek in 1987 which were unusually small and early (Table 1).

The mean weights and lengths of the F_2 hybrids were similar to those of the F_1's except for female lengths ($P < 0.10$); however, only three females returned, so the comparison is very weak (Table 2). Comparisons of variances were not made because the sample sizes of the F_2's were very small.

There were no differences between average meristic counts of pooled 1985 (F_1 and control) returns and 1987 (F_2) returns (Table 3). Comparisons of F_2 average counts with either F_1 or 1985 control fish revealed only the small excess in F_1 male gill raker count. However, there was more asymmetry in bilateral gill raker counts ($P = 0.048$) of F_2 fish (Table 4). The extent of bilateral asymmetry was even more noticeable in the proportion of F_2 fish asymmetrical for both gill rakers and branchiostegals ($P = 0.020$). All meristic counts were in the middle of the ranges reported as typical of pink salmon (Hart 1973).

Discussion

We recovered both F_1 and F_2 even- by odd-year hybrids and controls at the Auke Creek weir during the late run. Rates of return of F_1 and control fish were the same, but less than that estimated for naturally produced fish. The lower rate is adequately explained by fin-marking handicap (Ricker 1976) and hatchery effects (Bailey et al. 1976). In contrast, only 11 F_2 hybrids were recovered in 1987. The return rate (0.21%) was much lower than the rate estimated for naturally produced fish (4.07%), too great a difference to attribute to marking handicap. Although there was no hatchery control group, it is conceivable that poor incubation conditions and low fry quality could have reduced survival. However, a simple calculation of survivals from Table 1 can be made to account for hatchery effect and to estimate the relative survival of F_2 and wild (parental type) fish. In 1985, the survival of wild fry was 1.81 that

TABLE 3. Counts of gill rakers and branchiostegals in control (all parents mature in 1983), F_1 hybrids (dams mature in 1983 and sires mature in 1982), and F_2 hybrids (offspring of F_1's) and summary of counts for 1985 and 1987 returns. Two-tailed *t*-tests tested equality of bilateral counts ($^aP < 0.05$), between F_1 hybrid and control groups ($^bP < 0.05$ and $^cP < 0.01$), and between 1987 (pooled sexes) and 1985 (pooled sexes and control and F_1's) summaries (all $P > 0.10$).

	Females				Males			
	Left		Right		Left		Right	
	Control	F_1	Control	F_1	Control	F_1	Control	F_1
				Gill rakers				
Mean	30.30	30.48	30.37	30.41	30.04[b]	30.54[b]	29.88[c]	30.56[c]
n	30	27	30	27	48	66	48	66
Variance	1.183	0.644	1.551	1.020	1.700	1.298	1.261	1.266
				Branchiostegals				
Mean	11.50	11.57	11.50	11.57	11.50	11.74[a]	11.48	11.45[a]
n	32	30	32	30	48	66	48	66
Variance	0.323	0.530	0.387	0.461	0.426	0.533	0.425	0.344
				Summary				
	Gill rakers				Branchiostegals			
	Left		Right		Left		Right	
	1985	1987	1985	1987	1985	1987	1985	1987
Mean	30.35	30.00	30.31	30.09	11.60	11.36	11.49	11.82
n	171	11	171	11	176	11	176	11
Variance	1.311	0.800	1.242	0.491	0.469	0.454	0.388	0.765

TABLE 4. Bilateral asymmetry in gill raker and branchiostegal counts of hybrids between even-year males and odd-year females and for odd-year controls. Log-likelihood ratios (*G*-test) tested homogeneity between F_1 hybrids and controls and among 1985 returns ($^aP < 0.10$). There was no heterogeneity ($P > 0.10$) among 1985 returns. Exact probability (in parentheses) of the asymmetry observed for 1987 returns was computed from a binomial expansion assuming the frequencies observed in 1985.

			Proportion asymmetrical		
Sex	Group	*n*	Gill rakers	Branchiostegals	Both
Female	Control	30	0.500	0.600[a]	0.233
	F_1 hybrid	27	0.444	0.370[a]	0.148
Male	Control	48	0.479	0.354	0.229
	F_1 hybrid	66	0.606	0.500	0.242
	1985 return	171	0.526	0.456	0.222
	1987 return	11	0.818 (0.048)	0.636 (0.185)	0.545 (0.020)

of hatchery fry (0.0526/0.0291 = 1.81). In 1987, the survival of F_2 hybrid fry was 0.051 that of wild fry (0.0021/0.0407 = 0.051). By combining these terms to factor out the hatchery effect, the survival of the F_2's appears to be only 0.09 of the wild fish ($1.81 \times 0.051 = 0.09$). Pink salmon in Auke Creek have strong subpopulation structure defined in both time and space (Taylor 1980; Lane et al. 1990); the F_1's, controls, and F_2's returned during the late run; hybridization did not appear to affect time of return.

Withler and Morley (1982) conducted a similar experiment at Bear River on Vancouver Island, British Columbia, by combining cryopreserved milt taken from native fish in 1974 with ova transplanted from Glendale River on the mainland in 1975 and using fish produced with transplanted 1975 Glendale River gametes as a control. Their F_1 hybrids and controls were recovered in commercial catches, but none returned to the release site. R. B. Morley (Pacific Biological Station, Nanaimo, B.C., pers. comm.) suggests that olfactory cues may have been obscured by concrete work that took place at the time of imprinting but which was completed long before adults returned.

We hybridized even- and odd-year pink salmon populations to test the hypothesis that these genetically isolated populations had unique, coadapted genomes. Evidence of the uniqueness would be a reduction in survival of F_1 or F_2 hybrids or change in fitness-related traits. We measured survival as return to the release site. Fish that did not return were lost to the population.

In addition to having similar survival rates and return timing, the control and F_1 fish (partitioned by sex) had the same average weights and lengths. There were differences, however, between the variances of weights and lengths which were generally larger for the F_1 fish than for control fish. These results are not surprising because even- and odd-year pink salmon are quite distinct genetically (Aspinwall 1974; Johnson 1979; McGregor 1982; Beacham et al. 1988), even though the differences are not fixed allele differences (they are quantitative rather than qualitative). Hybridizing populations possessing quantitative differences rather than fixed differences increases genetic variation in the F_1's which results in a concomitant increase in the phenotypic variance. The increases in variance we observed, although modest, are consistent with the prediction. Average weights and lengths of returning F_2 males were similar to those

of F_1 males. There were too few returns to permit comparisons of F_1 and F_2 females or of variances between generations.

Disruption of coadapted allele complexes may result in anomalies in development (Graham and Felley 1985). One kind of character that can reflect such effects on development are meristic characters such as the number of vertebrae. Disturbances in development may be reflected by fluctuating asymmetry in paired bilateral counts of structures such as gill rakers or branchiostegals (Van Valen 1962). In our experiment, only F_1 males differed meristically; they averaged about one half an additional gill raker and had a fraction more of left than right branchiostegals. The reason for the difference is not clear, but it may reflect genetic differences between even- and odd-year lines or susceptibility to developmental environment expressed in males.

The proportion of fish that were asymmetrical for bilateral counts of gill rakers and branchiostegals was similar for F_1 and control fish, except for a small difference of asymmetry ($P < 0.10$) of branchiostegals of females. Although there were only 11 returning F_2 fish, they had significantly more asymmetry for bilateral gill raker counts ($P = 0.048$), and there was a much higher proportion of fish asymmetrical for both gill raker and branchiostegal counts ($P = 0.020$). One interpretation of these results is that disruption of coadaptive gene complexes in the F_2's had a disruptive effect on developmental canalization. Of course, adverse hatchery conditions could also produce developmental anomalies and no appropriate controls were available for examination in 1987. Because of the small return and the absence of a control group with which to compare them, it is not possible to determine whether or not the poor survival and apparent increase in developmental anomalies are a result of outbreeding depression; but the data are consistent with such a result.

It is unlikely that the effects we observed resulted from inbreeding or random drift. The number of parents used in 1983 to produce the F_1's was nearly 40, 20 males and 20 females. This is an effective population size (N_e) of about 40 which corresponds to an inbreeding coefficient ($F = 1/2N_e$) of about 0.0125. Because these parents shared no recent common ancestors, the effects of the inbreeding from this number would not be realized until the F_2 generation. We are aware of no situation where results as extreme as we observed resulted from an inbreeding coefficient so small.

Although the results of this test of the effects of hybridizing genetically isolated salmon populations are not conclusive, they are sufficiently convincing to suggest that outbreeding depression ought to be a real concern to managers of salmon enhancement and may result from hybridizing distinct populations. Outbreeding depressive effects of introgression of transplanted or differently coadapted hatchery fish into wild stocks may be insidious because outbreeding depression would probably not be apparent until the second generation, which for many salmon species is 6 or more yr later and too late to avert the effects of years of interbreeding. Our concern is heightened by the common practice of transplanting a successful hatchery stock rather than developing new ones from locally adapted stocks. Our evidence compells us to suggest that outbreeding depression can result from hybridization of distinct populations and that outbreeding depression ought to be a serious concern for managers of salmonid enhancement. Transplantations that are likely to result in the outbreeding of locally adapted fish are risky; they should be undertaken only with the most compelling reasons and should be carefully monitored beyond the first generation.

Acknowledgements

We thank J. Pella for advice in statistical analysis, J. Stoss for help with cryopreservation, and S. Hagen, K. Iverson, J. Taylor, M. Thomason, J. Joyce, C. Smoot, and S. Shirley. J. Gharrett, R. Morley, R. Reisenbichler, and two anonymous referees provided constructive criticism. The U.S. NMFS Auke Bay Fisheries Laboratory (ABL), University of Alaska, and the Alaska Department of Fish and Game cooperated in operation of the Auke Creek weir, and ABL and the Alaska Territorial Sportsmen supported our use of the Auke Creek Hatchery. This work was sponsored by the Alaska Sea Grant College Program, cooperatively sponsored by NOAA, Office of Sea Grant and Extramural Programs, Department of Commerce, under grant Nos. NA86AA-D-SG041, project No. R/02-11, and the University of Alaska with funds appropriated by the state.

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Induction of Foci of Cellular Alteration and Other Hepatic Lesions in English sole (*Parophrys vetulus*) Exposed to an Extract of an Urban Marine Sediment

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Epizootiological analyses of field data on prevalances of hepatic neoplasms and other hepatic lesions in English sole (*Parophrys vetulus*) from Puget Sound suggest a link between these lesions and sediment-associated xenobiotics, particularly aromatic hydrocarbons (AHs). To further investigate this relationship, English sole from a minimally contaminated site were parenterally exposed at monthly intervals for 1 yr to an organic solvent extract of a contaminated urban sediment containing high levels of AHs (30 or 75 mg/kg body weight) or to the model carcinogen benzo[a]pyrene (BaP, 12 mg/kg body weight). Control groups included English sole exposed to an extract of a relatively uncontaminated reference sediment, sole exposed to the carrier only, and untreated sole. Eighteen months after the initial injections, examination of livers revealed a significantly different (increased, $p \leq 0.05$) incidence of a spectrum of hepatic lesions, including presumptive preneoplastic foci of cellular alteration, in sole exposed to the urban sediment extract or BaP. Sole in control groups did not develop any of these hepatic lesions. These results represent the first direct demonstration of the hepatotoxicity of sediment-associated chemical contaminants in English sole and strengthen the case for a cause-and-effect relationship between exposure to AHs and epizootic neoplastic hepatic lesions in this species.

Des analyses épizootiologiques des données de terrain recueillies sur la présence de néoplasmes hépatiques et d'autres lésions hépatiques chez la sole anglaise (*Parophrys vetulus*) du Puget Sound révèlent qu'il peut y avoir un lien entre ces lésions et des éléments xénobiotiques associés aux sédiments notamment les hydrocarbures aromatiques (HA). Pour étudier ce lien, on a exposé par administration parentérale, à intervalles mensuels pendant un an, des soles anglaises provenant d'un site très peu contaminé à un extrait sur solvent organique d'un sédiment urbain contaminé contenant de fortes concentrations de HA (30 ou 75 mg/kg de poids corporel) ou au cancérogène modèle benzo[a]pyrène (BaP, 12 mg/kg de poids corporel). Les groupes témoins comprenaient des soles anglaises exposées à un extrait de sédiment de référence relativement peu contaminé, des soles exposées seulement au support et des soles non traitées. Dix-huit mois après les premières injections, l'examen des foies a révélé une incidence significativement différente (augmentation, $p \leq 0,05$) d'une gamme de lésions hépatiques, notamment des foyers d'altération cellulaire à caractère présumément prénéoplasique, chez les soles exposées au sédiment urbain ou au BaP. Les poissons des groupes témoins ne présentaient aucune de ces lésions hépatiques. Ces résultats constituent la première démonstration directe de l'hépatotoxicité des polluants chimiques associés aux polluants chez la sole anglaise, et renforcent l'hypothèse de la relation de cause à effet entre l'exposition aux HA et les néoplasmes hépatiques à caractère épizootique chez cette espèce.

Received August 22, 1990
Accepted March 13, 1991
(JA695)

Reçu le 22 août 1990
Accepté le 13 mars 1991

There is a rapidly expanding body of evidence linking exposure to the myriad chemical contaminants found in the urban bays, rivers, and estuaries of North America to high prevalences of hepatic lesions, including neoplasms, in bottom-dwelling fish. Hepatic neoplasms have been reported in English sole (*Parophrys vetulus*) residing in urban bays of Puget Sound (McCain et al. 1977, 1982; Pierce et al. 1978; Malins et al. 1984, 1985a, 1985b; Myers et al. 1987), Atlantic tomcod (*Microgadus tomcod*) from the Hudson River Estuary (Smith et al. 1979), brown bullhead (*Ictalurus nebulosus*) in waterways associated with the Great Lakes (Baumann et al. 1982; Black 1983), winter flounder (*Pseudopleuronectes americanus*) from Boston Harbor (Murchelano and Wolke 1985), mummichog (*Fundulus heteroclitus*) from the Elizabeth River in Virginia (Vogelbein et al. 1990), and white croaker (*Genyonemus lineatus*) in coastal waters adjacent to Los Angeles (Malins et al. 1987).

Findings from a number of laboratory and field studies also support a relationship between exposure to chemical contami-

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nants and hepatic neoplasms. For example, hepatocellular and cholangiocellular carcinomas histologically similar to those reported in field studies have been produced in aquarium fishes such as the guppy (*Poecilia reticulata*) by exposure to 4-dimethylaminoazobenzene, *N*-2-fluorenylacetamide, *o*-aminoazotoluene, diethylnitrosamine, and dimethylnitrosamine (Pliss and Khudoley 1975) and 7,12-dimethylbenz[*a*]anthracene (DMBA) (Hawkins et al. 1989). Hepatic neoplasms have also been induced by exposures to DMBA in two livebearers, *Poeciliopsis lucida* and *P. monacha* (Schultz and Schultz 1982). Other examples include the demonstration of the carcinogenicity of methylazoxymethanol acetate (Aoki and Matsudaira 1977) and diethylnitrosamine (Ishikawa et al. 1975) in medaka (*Oryzias latipes*) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Hendricks et al. 1980), dimethylnitrosamine (Ashley and Halver 1968; Grieco et al. 1978), and benzo[*a*]pyrene (BaP) (Hendricks et al. 1985) in rainbow trout (*Oncorhynchus mykiss*). The carcinogenicity of BaP is particularly noteworthy, as this compound is a frequently detected contaminant of sediments in urban waterways. Indeed, results of field studies conducted in Puget Sound over the past several years by our laboratory have consistently demonstrated a highly significant statistical association between prevalences of hepatic neoplasms in English sole and sediment concentrations of aromatic hydrocarbons (AHs) at the site of capture (Malins et al. 1984; Myers et al. 1987). Moreover, laboratory studies have shown that BaP can be activated to intermediates that form adducts with English sole hepatic DNA and that these adducts are persistent for several months (Varanasi and Gmur 1981; Nishimoto and Varanasi 1985; Varanasi et al. 1989a). The formation of DNA adducts is considered to be a critical early event in the multistep pathway of chemical carcinogenesis.

To date there has been limited laboratory investigation of the hepatotoxicity (including hepatocarcinogenicity) of complex mixtures of organic compounds typical of those found associated with sediments of urban waters (Black et al. 1985; Metcalfe et al. 1988). Even fewer studies have focused on the induction of lesions in a feral fish species which is naturally at risk for neoplastic liver disease. Accordingly, the present investigation was undertaken to assess the hepatotoxicity in English sole of an organic solvent extract of a naturally occurring marine sediment contaminated with high concentrations of aromatic compounds. Also tested was the model hepatocarcinogen BaP. Reported here are the results of histological examinations of liver from English sole following a 1-yr exposure and 6-mo holding period, confirming the hepatotoxicity and capacity of both the sediment extract and BaP to induce presumptive preneoplastic foci of cellular alteration in this species.

Materials and Methods

Experimental Animals, Holding Conditions, and Diet

Two-year-old English sole (mean weight 57 ± 14 g) were captured by otter trawl in Useless Bay, a nonurban site in northern Puget Sound (Washington State). Fish collected at this location exhibited extremely low levels of fluorescent aromatic compounds (FACs) in bile (an indicator of exposure to AHs) (Krahn et al. 1986), and gross and microscopic examination of representative fish revealed no detectable lesions in liver, kidney, gill, spleen, small intestine, or gonads. Moreover, sediments from this site have been historically free of contamination, and chemical analysis of sediment collected at the time of fish capture showed nondetectable concentrations of aromatic and chlorinated hydrocarbons (detection limits were 10 and 1 ng/g dry weight, respectively).

English sole were maintained in the laboratory in 150-L rectangular glass aquaria receiving flowing seawater at 4 L/min. Seawater was obtained from Puget Sound at a depth of 10 m and passed sequentially through sand, woven fiber, and activated charcoal filters to remove particulates and most waterborne organic contaminants. All seawater was UV treated in an attempt to minimize the introduction of opportunistic fish pathogens. Fish were maintained under constant white fluorescent light at ambient seawater temperature and salinity, which varied seasonally from 6 to 16°C and from 22 to 30‰, respectively.

Fish were fed daily a pelletized diet at a rate of 2% of body weight/d. The diet consisted of 40% clam/krill mixture (1:1), 32% fish meal, 20% wheat starch, 4% fish oil, 2% mineral mix, and 2% vitamin mix. Dry weight analysis was 7.3% ash, 14.3% oil, 36.2% protein, and 42.2% carbohydrate. The diet, which was developed specifically for this study, was prepared at 3-mo intervals, vacuum packed in 1-kg aliquots, and stored at −20°C until used. Prior to synthesis, the raw dietary components were analyzed for xenobiotics and were found to be essentially free of, or to contain background levels of, chlorinated and aromatic hydrocarbons. The diet was also analyzed by electron spin resonance spectroscopy and was negative for free radical activity.

Test fish were acclimated to laboratory conditions for 3 mo prior to exposure. During this period, all fish received prophylactic treatments with the broad-spectrum antibiotic Furanace® (6-hydroxymethyl-2-{2-{5-nitro}2-furylvinyl} pyridine; ten 1-h bath treatments at a concentration of 0.1 mg/L) and the ectoparasitic agent formalin (a single 1-h bath treatment at a concentration of 250 ppm). Earlier attempts to hold English sole for extended periods had been unsuccessful because of high mortalities associated with bacterial septicemias and infestations of protozoan and metazoan parasites, even with UV treatment of seawater. However, over the 3-mo acclimation period following these treatments, only a 16% mortality occurred, all of which could be attributed to post-capture stress and low-level ectoparasitic infestations (mostly *Gyrodactalus* sp.). There was no evidence of other infectious disease.

Chemicals and Sediment Extracts

BaP (CAS:50-32-5) of approximately 98% purity was purchased from Sigma Chemical (St. Louis, MO) and further purified by silica chromatography as described by Varanasi and Gmur (1981).

Sediments for extraction and testing were collected at Eagle Harbor on Bainbridge Island (central Puget Sound) and West Beach on Whidbey Island (northern Puget Sound). Eagle Harbor is known to be heavily contaminated with creosote hydrocarbons, and English sole residing in this embayment exhibit some of the highest prevalences of hepatic neoplasms and related disorders observed at any location in Puget Sound (Malins et al. 1985b; Myers et al. 1987). The Eagle Harbor site is unusual in that despite the high levels of AHs, other xenobiotics, such as chlorinated hydrocarbons and most heavy metals, are found at extremely low levels. In contrast, West Beach is located in a rural area of the Puget Sound basin and the sediment is essentially free of detectable chemical contaminants (e.g.

TABLE 1. Concentrations of selected aromatic and nitrogen-containing aromatic compounds in fraction *S3* prepared from Eagle Harbor sediment extract (EHSE). n.d. = not detected; detection limits for AHs and NCACs were 3.5 and 4.2 μg/mL, respectively.

Compound	μg/mL[a]
Aromatic hydrocarbons	
Naphthalene	n.d.
2-Methylnaphthalene	n.d.
1-Methylnaphthalene	21
Biphenyl	14
2,6-Dimethylnaphthalene	50
Acenaphthene	300
Fluorene	330
Phenanthrene	950
Anthracene	200
Fluoranthene	2300
Pyrene	900
Benz[*a*]anthracene	1900
Chrysene	2400
Benzo[*e*]pyrene	500
Benzo[*a*]pyrene	500
Perylene	140
Dibenz[*a*,*h*]anthracene	60
Nitrogen-containing aromatic compounds	
7-and/or 6-Methylquinoline	3.5
2,7-and/or 2,6-Dimethylquinoline	2.4
2,8-Dimethylquinoline	7
2,4-Dimethylquinoline	n.d.
1-Cyanophthalene	140
2-Cyanophthalene	6
7,8-Benzoquinoline	n.d.
Acridine	55
Carbazole	220
2-Methyl-benzo[*h*]quinoline	45
1-Methylcarbazole	70
3-Methylcarbazole	60
2-and/or 4-Methylcarbazole	60
Benzo[*a*]carbazole	80

[a]Values are for the high-dose parenteral exposure group of English sole given 75 mg/kg b.w. of EHSE.

< 2.5 and 3.0 ng/g dry weight AHs and nitrogen-containing aromatic compounds (NCACs), respectively).

Organic solvent extracts of these sediments were prepared essentially as described by Krone et al. (1986). Briefly, extracts were prepared by placing 500 g of sediment, 2000 mL of methylene chloride, and 1500 g of sodium sulfate in a 4-L glass bottle and rolling on a rock tumbler at about 1000 rpm for 18 h. The methylene chloride was decanted into Erlenmeyer flasks. An additional 2000 mL of methylene chloride was added to the 4-L bottle and the rolling repeated for 6 h. All methylene chloride extracts were combined, concentrated to 10 mL in Erlenmeyer flasks fitted with three-ball Synder columns, and the solvent exchanged to hexane. Starting with 10 kg of each test sediment, a total of 25.0 and 0.134 g, respectively, of Eagle Harbor sediment extract (EHSE) and West Beach sediment extract (WBSE) was obtained.

Sediment extracts were fractionated by silica-alumina column chromatography as described by Krone et al. (1986). The columns (47-mm inside diameter glass columns with 1-L reservoirs) were dry packed with 100 g of alumina and 200 g of silica and washed with 1 L of pentane. The sediment extract in hexane (from 500 g of sediment) was applied to the column and allowed to drain. The column was then washed sequentially with 1 L of pentane, 1 L of 2% methylene chloride in pentane, 200 mL of methylene chloride, and, finally, with 800 mL of 20% methanol in methylene chloride. The latter two fractions (methylene chloride and 20% methanol in methylene chloride) were combined and concentrated to produce the S3 fraction. The S3 fraction of the EHSE, which was selected for hepatotoxicity testing, was enriched in high molecular weight AHs (four to six benzenoid rings) and NCACs (Table 1). Concentrations of these compounds in the S3 fraction of WBSE were below limits of detection. The S3 fraction was dissolved in acetone and mixed with an equal volume of Emulphor EL-620 (polyoxyethylated castor oil; GAF Corp., New York) for injection.

Toxicokinetics

Time-course studies were conducted to determine the distribution and clearance of BaP, both alone and in the presence of EHSE, so as to provide a basis for selection of an exposure interval. Groups of 30 English sole were injected intramuscularly at 0 time with 75 or 30 mg EHSE/kg body weight (b.w.) or 15 mg BaP/kg b.w. containing trace levels of generally labeled [^{3}H]BaP (70 Ci/mmol(1 Ci = 37 GBq; Amersham, Arlington Heights, IL). The persistence of the BaP-derived radioactivity (and presumably most AHs) in liver, bile, and blood was determined after 8 h and 1, 2, 7, 14, 21, 28, and 60 d. At each sampling, three to seven sole were killed in each treatment group and tissues collected and stored at −65°C. Prior to determination of radioactivity, liver tissue was solubilized with Soluene-350 (Packard Instrument Co., Downers Grove, IL) by previously described methods (Varanasi and Gmur 1981), and blood was decolorized by treatment with isopropanol and hydrogen peroxide. Total radioactivity was determined in a toluene-based cocktail using a Packard model 300C liquid scintillation spectrophotometer.

Peak levels (percent dose) of BaP-derived radioactivity in liver and blood were detected within the first 8 h after exposure, while peak levels in bile were not observed until approximately 2 d postexposure (Fig. 1). The highest concentrations of radioactivity were detected in bile; levels of radioactivity in liver tissue were approximately 2 orders of magnitude less than those in bile. The level of BaP-derived radioactivity in blood was approximately an order of magnitude lower than that observed in liver. In all tissues examined, radioactivity declined rapidly over the first 14 d, followed by a much slower decline to 28 d and later. Of particular note, the tissue disposition and time-dependent loss of BaP-derived radioactivity was not affected by concurrent exposure to EHSE. Furthermore, these data demonstrated little change in the concentration of radioactivity in liver tissue between 14 and 60 d. Based on these findings, an exposure protocol consisting of injections every 4 wk was selected.

Long-Term Exposure

Groups of 32–35 English sole, divided between duplicate aquaria, were exposed by intramuscular injection over a 1-yr period to the S3 fractions of EHSE (two doses, 75 and 30 mg/kg b.w.) or WBSE or BaP as outlined in Table 2. The injection site was near the caudal peduncle on the eyed side. Controls included solvent-treated and uninjected groups of sole. Concentrations of the test solutions were adjusted such that fish received an injected volume of 1 μL/g b.w. The use of intramuscular injection was based on the demonstration of rapid

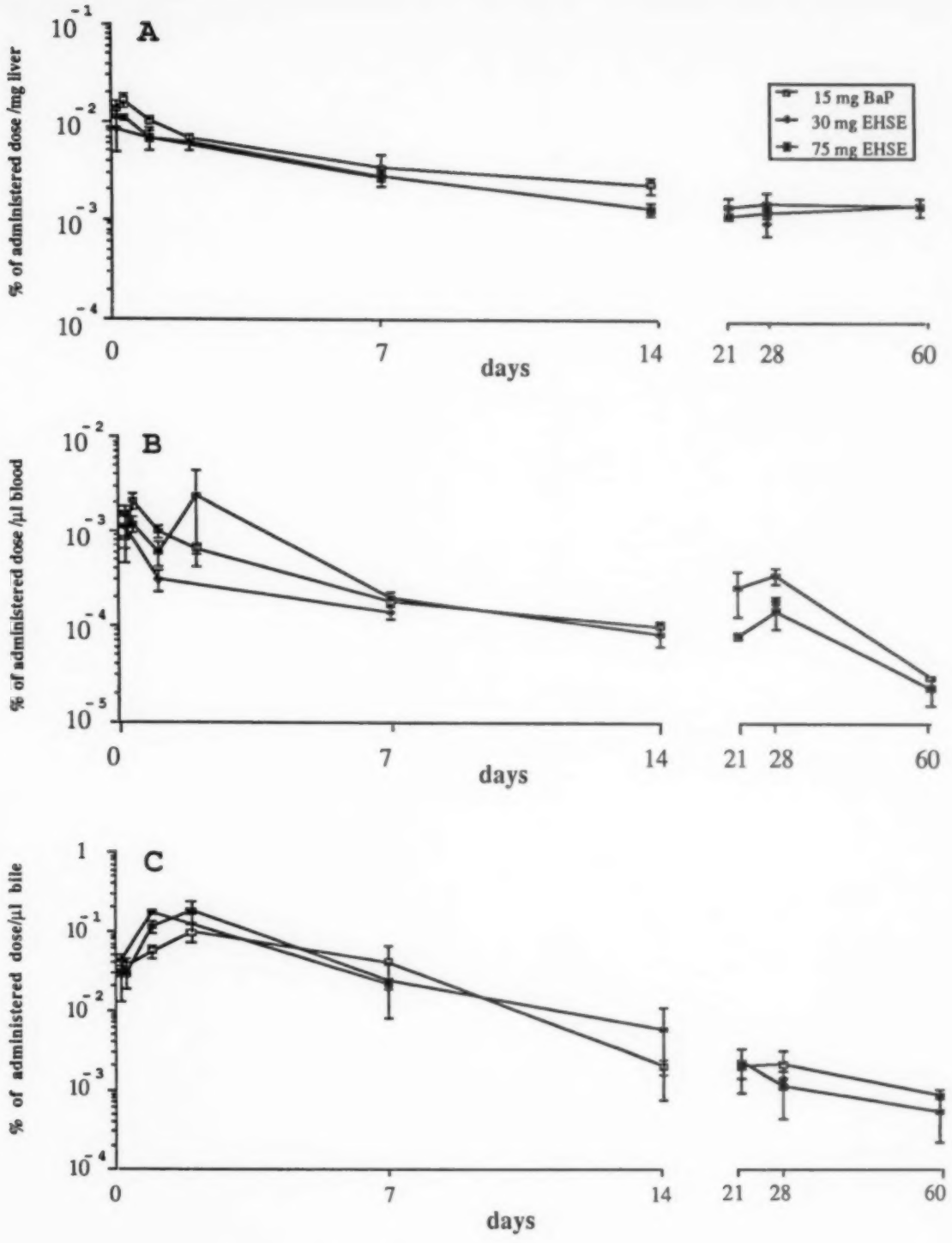

FIG. 1. Levels of BaP-derived radioactivity in English sole tissues after intramuscular injection of 75 mg of EHSE, 30 mg of EHSE, or 15 mg of BaP/kg b.w. each containing trace amounts of [^{3}H]BaP. Values are means ± SE (N = 3–7). (A) Liver; (B) blood; (C) bile.

TABLE 2. Summary of treatments and selected biological parameters among English sole exposed to Eagle Harbor and West Beach sediment extracts (EHSE and WBSE, respectively). Numbers and weights of fish are values at the start of exposure; survival, condition factors, liver somatic indices, and sex ratios are ending values.

Treatment	Dose/kg b.w.	Number of fish	Average weight (g)[a]	Survival (%)	Condition factor[b]	Hepatosomatic index	Sex ratio (male/female)
EHSE	75 mg	33	60.5 (2.4)	87.9	8.9 (0.14) × 10^{-3}[d]	1.14 (0.05)	18/11
EHSE	30 mg	35	58.1 (2.6)	65.7	8.8 (0.17) × 10^{-3}[d]	1.22 (0.08)	12/11
WBSE	0.3 mg[c]	34	53.9 (2.1)	88.2	9.4 (0.14) × 10^{-3}	1.22 (0.07)	18/12
BaP	12 mg	34	54.8 (2.0)	88.2	9.1 (0.24) × 10^{-3}	1.38 (0.08)	16/14
Carrier-solvent	1 mL	34	54.9 (2.3)	85.3	9.3 (0.13) × 10^{-3}	1.08 (0.05)	19/10
Uninjected controls	—	32	59.7 (2.5)	68.8	10 (0.21) × 10^{-3}	1.28 (0.06)	12/10

[a]Mean (standard error).
[b]Condition factor = [weight (g)/total length (cm)^3], mean (standard error).
[c]The concentration of WBSE was selected such that it reflected a volume of sediment extracted equal to that extracted to produce the high-dose concentration of EHSE.
[d]Significantly different ($p \leq 0.05$) from uninjected controls.

uptake and persistence of intramuscularly injected [^{3}H]BaP in the toxicokinetic experiment and the lack of any obvious leakage at the injection site when using the volume to body weight ratio we employed. Over the course of 1 yr the fish received 13 injections at 4-wk intervals. Fish were weighed and measured at this same interval.

Following the 1-yr exposure phase, the fish were held an additional 6 mo, at which point they were killed by severing the spinal cord. The abdominal cavity was opened, and any grossly visible abnormalities were noted. The entire livers were removed, weighed, and portions for histologic examination were routinely and uniformly excised from the central region of the liver along the longitudinal axis, to include the anterior lobe, the largest posterior lobe, and any grossly visible liver lesions. Other tissue collected included anterior and posterior kidney, spleen, gills, pancreas, and gonads. The tissues were fixed in Dietrich's fixative (Gray 1975), processed and embedded in paraffin, sectioned at 5 μm, and routinely stained with Mayers' hematoxytin and eosin–phloxine (Luna 1968).

All slides were examined microscopically without knowledge of treatment regime. A single tissue section from the routinely excised portion of the liver as well as sections from any grossly visible lesions were examined in each fish. Hepatic lesions were classified according to the previously described diagnostic criteria of Myers et al. (1987).

Results

Growth and Mortality

Survival of English sole over the 12-mo exposure and 6-mo holding periods was between 85 and 88% in all treatments except the uninjected controls and the 30 mg EHSE/kg b.w. (low-dose EHSE) exposure (Table 2). Losses among the control and low-dose EHSE group were 31 and 34%, respectively, and occurred predominantly in only one of the duplicate sets of aquaria. These losses were attributed to an infectious disease of unknown nature.

Fish in all groups increased in weight over the first 9 mo of exposure, at which time growth slowed and remained relatively level (Fig. 2). However, in the EHSE exposure groups (both high and low doses), cumulative weight began to decline at the 9-mo point. At the 12-mo point, when the active exposure phase ended, sole in all groups began gaining weight again. From the length and weight data collected at the completion of the 18-mo experiment, condition factors [CF = (weight in grams)/(total length in centimetres)^3] were calculated for each treatment group (Table 2). Statistical comparisons of the CFs among treatment groups and by gender using two-way ANOVA indicated that female sole in all groups had a significantly different (higher, $p = 0.004$) CF than males. Moreover, as suggested by growth pattern (Fig. 2), mean CFs were significantly different (higher, $p = 0.05$) in the uninjected controls and the WBSE-exposed fish as compared to the EHSE-exposed sole (both doses) (Newman-Keuls multiple comparison test).

Hepatosomatic indices (HSIs, the ratio of liver weight to body weight × 100) were also calculated at termination of the experiment (Table 2). Statistical comparisons among treatment groups and with respect to gender revealed no significant association between HSIs and gender (ANOVA, $p = 0.146$). In contrast, there was an apparent significant relationship between HSI and treatment (ANOVA, $p = 0.026$); however, subsequent statistical testing for differences among specific treatment pairs failed to identify where significant differences occurred.

Although all treatment groups contained a higher percentage of males (Table 2), the randomness of gender composition among treatment groups was confirmed statistically (χ^2, $p = 0.90$).

Hepatic Lesion Incidences

Eleven distinct types of histological conditions or lesions were detected in the 161 sole livers examined (Table 3). Three types of putatively preneoplastic foci of cellular alteration (FCA) were detected: clear cell focus, eosinophilic focus, and basophilic focus. The clear cell focus was composed of vacuolated hepatocytes with minimal nuclear alteration arranged in spherical, noncompressing micronodules. The chief cytologic features of the eosinophilic focus were slight hepatocellular hypertrophy and increased cytoplasmic eosinophilia with a granular texture. Basophilic foci (Fig. 3) featured a hyperbasophilic cytoplasm in normal-sized hepatocytes with varying degrees of nuclear atypia. All types of FCA were arranged in discrete micronodular, proliferative foci and the borders of each focus blended indistinctly with the surrounding hepatic tubules, with compression being minimal or, most commonly, absent.

The necrotic and degenerative hepatocellular conditions (liver necrosis) were characterized by pyknosis, cytoplasmic

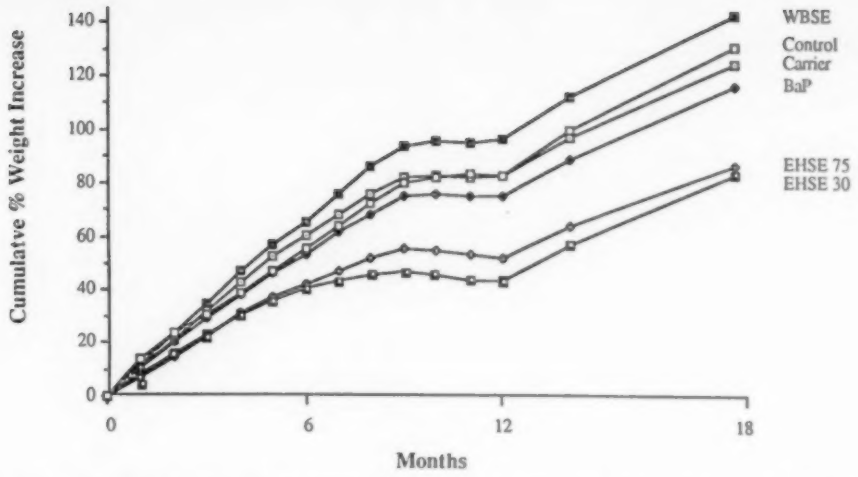

FIG. 2. Cumulative percent weight increase of English sole parenterally exposed to EHSE, WBSE, BaP, or carrier and uninjected controls. Sole were injected at 4-wk intervals for a 12-mo period which was followed by a 6-mo holding period.

TABLE 3. Incidence (%) of hepatic conditions or lesions in English sole parenterally exposed to Eagle Harbor sediment extract (EHSE, 30 or 75 mg/kg) or benzo[*a*]pyrene (BaP). Separate control groups received an injection of extract from the reference sediment from West Beach (WBSE), solvent carrier only, or were uninjected. Data from the three control groups were combined for statistical analyses. *Significantly different than all (combined) controls at $p \leq 0.05$ (Fisher's exact test); **Significantly different than all (combined) controls at $p \leq 0.005$ (Fisher's exact test); ***Significantly different than all (combined) controls at $p \leq 0.0005$ (Fisher's exact test).

	Controls			Combined	Treatments		
Lesion type	Uninjected	Carrier	WBSE	controls	BaP	EHSE 30	EHSE 75
Foci of cellular alteration	0.0	0.0	0.0	0.0	13.3**	13.6*	13.8*
Clear cell foci	0.0	0.0	0.0	0.0	0.0	9.1*	0.0
Eosinophilic foci	0.0	0.0	0.0	0.0	6.7	0.0	0.0
Basophilic foci	0.0	0.0	0.0	0.0	10.0*	9.1*	13.8**
Liver necrosis	4.5	0.0	3.3	2.5	16.7*	9.1	27.6***
Spongiosis hepatis	0.0	3.6	3.3	2.5	6.7	0.0	3.4
Fatty change	0.0	0.0	0.0	0.0	0.0	9.1*	17.2*
Hemosiderosis	0.0	0.0	0.0	3.3	3.3	0.0	17.2**
Nuclear pleomorphism	0.0	0.0	0.0	0.0	40.0***	22.7***	82.8***
Megalocytic hepatosis	0.0	0.0	0.0	0.0	10.0*	9.1*	58.6***
Hepatocellular regeneration	4.5	3.6	3.3	3.8	10.0	27.2**	41.4***
Biliary proliferation	0.0	3.6	0.0	1.3	6.7	0.0	10.3
Number examined	22	28	30	80	30	22	29

hyalinization, hydropic degeneration, and/or coagulative necrosis. Two types of lesions characterized by cytoplasmic storage of abnormally high amounts of either hemosiderin and other iron-containing pigments (hemosiderosis; Fig. 4) or lipid (fatty change) were also found. Nuclear pleomorphism (NP) was characterized by nuclear enlargement and heterogeneous nuclear shape in affected hepatocytes (Fig. 4 and 5). A unique degenerative condition, megalocytic hepatosis (MH) (Fig. 4 and 5), featured both marked nuclear and cellular enlargement along with hydropic changes and cytoplasmic hyalinization. Islands of hepatocellular regeneration characterized by small, basophilic hepatocytes arranged in nonspherical patterns were also found, generally accompanying liver necrosis and/or MH (Fig. 4).

Other types of lesions included a proliferative lesion involving the bile ductules, manifested by hyperplasia of these anatomical units, and spongiosis hepatis, characterized by cystic regions resulting from loss of parenchymal cells, which was observed rarely.

Several types of these lesions or conditions were detected exclusively in the livers of fish exposed to either EHSE (one or both doses) or BaP. Lesions detected exclusively in fish from all three treatment groups included basophilic foci (9.1–13.8%), MH (9.1–58.6%), and NP (22.7–82.8%). Lesions detected only

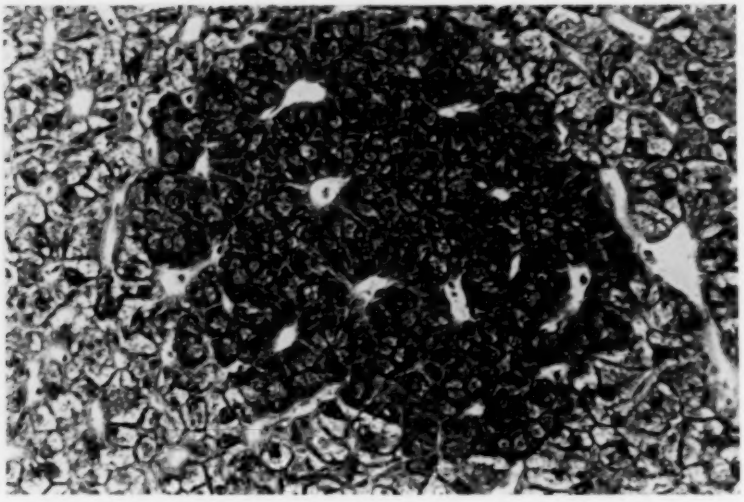

FIG. 3. Section of liver from an English sole exposed to 75 mg EHSE/kg b.w. showing a basophilic focus. The hepatocytes surrounding the focal lesion display nuclear pleomorphism. H&E, 256×.

FIG. 4. Section of liver from an English sole exposed to 75 mg EHSE/kg b.w. Lesions shown include hepatocellular hemosiderosis (small arrows), megalocytic hepatosis featuring hepatocellular hypertrophy and nuclear enlargement, nuclear pleomorphism, and densely cellular islands of hepatocellular regeneration (large arrow). H&E, 256×.

in fish exposed to EHSE were clear cell foci (9.1% in low-dose treatment) and fatty change (9.1 and 17.3% in low- and high-dose treatments, respectively). Hepatocellular hemosiderosis was detected in sole in the BaP (3.3%) and high-dose EHSE (17.3%) treatments. Hepatocellular regeneration (10.0–41.4%) and liver necrosis (9.1–27.6%), while not restricted to the EHSE and BaP treatment groups, were present at higher incidences in these groups than in controls or the carrier and WBSE treatment groups.

Statistical analyses of these data revealed significantly higher (Fishers exact test, $p \leq 0.05$) incidences of liver necrosis, fatty change, hemosiderosis, NP, MH, hepatocellular regeneration, and FCA in sole exposed to the high-dose EHSE when compared with combined controls (i.e. uninjected, carrier-injected, and WBSE-exposed fish) (Table 3). Significantly higher incidences of fatty change, NP, MH, hepatocellular regeneration, and FCA were also evident in the low-dose EHSE-exposed sole compared with the same controls. Moreover, significantly higher incidences of liver necrosis, NP, MH, and FCA were detected in BaP-exposed sole relative to controls. The incidences of basophilic foci (one of the three types of FCA detected) were significantly higher in English sole exposed to either dose of EHSE or BaP when compared with combined controls (Table 3). Finally, the influence of gender on lesion

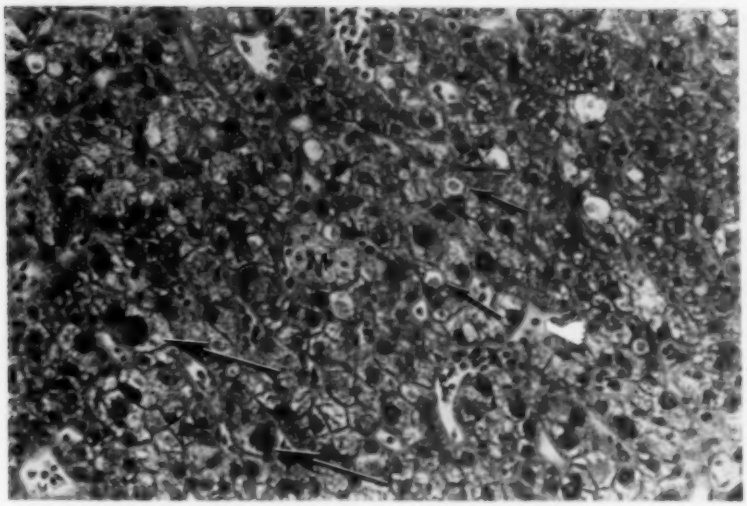

FIG. 5. Section of liver from an English sole exposed to 75 mg EHSE/kg b.w. Lesions shown include hepatocellular cytoplasmic hyalinization (small arrows), megalocytic hepatosis featuring cellular hypertrophy and marked hepatocellular nuclear enlargement (large arrows), and a generalized pleomorphism of hepatocellular nuclei. Also shown is a macrophage aggregate (M). H&E stain, 256×.

occurrence within treatment groups was statistically tested using two-way contingency analyses ($p \leq 0.05$). Results showed distribution and incidence of lesion types to be independent of gender.

Lesion Incidences in Other Organs

Three types of lesions in the posterior kidney were found at significantly higher incidences in fish exposed to either BaP or the high dose of EHSE. Tubular necrosis, characterized by pyknosis and coagulative necrosis of the tubular epithelium with exfoliation into the lumen, and NP of the tubular epithelium were found at significantly higher ($p \leq 0.05$) incidences (51.7 and 48.3%, respectively) in sole exposed to the high-dose EHSE compared with those found in sole exposed to carrier only (24.1% incidence of each lesion). Incidences of tubular proliferation, distinguished by irregular, thickened profiles of tubular epithelial cells containing pleomorphic nuclei, were significantly higher ($p \leq 0.05$) in sole exposed to BaP (43.8%) and the high dose of EHSE (51.7%) than in fish exposed to carrier only (26.0%).

No lesions were detected in spleen, gill, small intestine, anterior kidney (the site of hematopoiesis in fish), or pancreas in any of the treatment groups. Gonads also were normal except for the presence of a high proportion of atretic follicles in most of the vitellogenic females; incidence was unrelated to treatment or group.

Discussion

The results of the present investigation represent the first direct demonstration of the hepatotoxicity in English sole of a common sediment contaminant, BaP, and of a complex mixture of chemical contaminants present in an urban marine sediment. English sole exposed over a 12-mo period to either BaP or EHSE, followed by a 6-mo holding period, developed a spectrum of degenerative, regenerative, and focal proliferative hepatic lesions or conditions. The most compelling evidence for the hepatotoxicity of both the BaP and EHSE was the significantly elevated incidences of NP, MH, hepatocellular regeneration, and presumptive preneoplastic FCA. Control English sole, including those in the uninjected group and the WBSE and carrier-only groups, either did not develop these same lesions or, in the case of hepatocellular regeneration, developed them at significantly lower incidences.

The spectrum of lesions induced in this study was indistinguishable from that observed in sole captured in Eagle Harbor (Malins et al. 1985a, 1985b; Myers et al. 1987), the location where the test sediment was collected. Moreover, these lesions were typical of those observed among English sole residing in contaminated urban areas throughout the Puget Sound Basin (Malins et al. 1984). Of particular note among the lesions induced by both BaP and EHSE exposure was the basophilic focus, a lesion which has previously been shown not only to possess preneoplastic potential in rodents (Bannasch et al. 1982; Farber 1980; Williams 1980), but the lesion most proximate to frank hepatocellular carcinomas in the rat hepatocarcinogenesis model (Williams 1982). That the basophilic focus and hepatocellular neoplasms are also temporally related in English sole has recently been suggested by Myers et al. (1987) based on analysis of lesion cooccurrence in sole from Eagle Harbor. Additional evidence for a relationship between these lesions is the extensive work of Bailey, Hendricks, and co-workers in the rainbow trout hepatocarcinogenicity model in which the basophilic focus is considered a microcarcinoma or carcinoma in situ (Hendricks et al. 1984).

The nature of the specific organic compound(s) in the EHSE that were responsible for the hepatotoxicity and, in particular, the development of basophilic foci in exposed English sole cannot be determined from these experimental data. While the test fraction was prepared such that it was enriched in high molecular weight AHs and NCACs (14:1, by weight), the sum of the

measured analytes in these two chemical classes represents only approximately 16% of the total mass of the tested fraction. Therefore, the possibility exists that during fractionation of the EHSE, which was done to reduce the concentration of the more acutely toxic, lower molecular weight AHs, certain compounds essential for neoplastic transformation or promotion were inadvertently removed. However, based on similar patterns of lesion cooccurrence in both the EHSE- and BaP-exposed fish, as well as the demonstration in other studies that DNA-AH adducts are detectable in English sole parenterally exposed to both EHSE and BaP (Varanasi et al. 1989a, 1989b), it is tempting to speculate that carcinogenic AHs present in EHSE may play a critical role in lesion induction. Since BaP per se was a relatively minor component of the aromatic compounds in EHSE, a variety of different AHs or NCACs may be acting individually or synergistically to induce hepatic lesions.

The failure to produce frank neoplasms with BaP in English sole in the present study contrasts with recent findings of Hendricks et al. (1985) showing 25 and 46% incidence of hepatocellular carcinoma at 18 mo in the Shasta strain of rainbow trout following exposure to BaP by either feeding or repeated injection, respectively. Also, Black et al. (1988) reported induction of hepatic tumors (4.3 to 15.9%) in 1-yr-old Shasta rainbow trout exposed in ovo to BaP. Although a variety of factors, including species-specific metabolic differences, genetic variation within species, route of exposure, dose, age at initiation of exposure, diet, and temperature (see Bailey et al. 1989 for review), may contribute to this difference in tumor response between trout and the sole in this study, several factors are worth discussing in greater detail.

The dose of BaP administered (12 mg/kg b.w.) and the exposure route used in the present study were selected on the basis of results of a preliminary toxicokinetic study showing high and persistent liver levels of BaP or its metabolites following intramuscular injection of a similar dose (15 mg BaP/kg b.w.) and on the basis of the solubility of BaP in the acetone/Emulphor carrier. Although growth was inhibited in the groups exposed to EHSE, the overall growth pattern and high survival of all groups suggest the appropriateness of the exposure regime. Exposures of longer duration or higher doses would likely have resulted in wasting or poor survival of the EHSE-exposed fish.

Both Hendricks et al. (1985) and Black et al. (1988) utilized carriers or exposure methods different from those used here (approximately 20 mg/kg × 12 and up to 200 mg/kg × 1, respectively). Possibly more important, however, might have been the use of the Shasta strain of rainbow trout which has been shown to be more sensitive to selected carcinogens than other strains of this species. In contrast, the present investigation was conducted with an apparently outbred, genetically heterogeneous population of wild fish. Regardless of the possible species differences, the degree of hepatic DNA adduct formation following exposure to BaP is comparable for both English sole and the Shasta strain of rainbow trout (Varanasi et al. 1986). Moreover, recent studies suggest that neither rainbow trout nor English sole exhibit appreciable DNA repair activity (based on persistence of bulky DNA adducts), particularly in comparison with rodents (Bailey et al. 1988; Varanasi et al. 1989a).

Although it is well known that hepatic tumors can be induced in less than 9 mo. in trout exposed to strong hepatocarcinogens such as aflatoxin B_1 (AFB_1), and that sensitivity to selected carcinogens peaks at about 5 mo of age (Bailey et al. 1989), no such information from laboratory studies exists on kinetics of chemical carcinogenesis in English sole. With regard to age at exposure, the inability to maintain healthy laboratory populations of young English sole (<2 yr old) precluded testing at a younger age. Moreover, based on epizootiological analyses of hepatic lesion (including neoplasms) prevalence in English sole from Puget Sound (Rhodes et al. 1987), the period between initiation and expression of frank hepatic neoplasms may be substantially longer in sole than in trout. For example, very few hepatocellular neoplasms are detected in wild English sole less than 2 yr of age. Although comparisons between field and laboratory studies should be made with caution, one interpretation of these data is that hepatocellular neoplasms in English sole typically take 2 or more yr to develop.

Yet another factor potentially influencing the lack of a frank tumor response in English sole was dietary protein level (Bailey et al. 1989). The diet developed for this study contained 36% protein and supported good growth and survival. However, Bailey et al. (1989) reported that in rainbow trout, carcinogenic response to AFB_1 increased from 33 to 90% by increasing the percentage of dietary protein from 40 to 70% in an isocaloric diet. These findings in trout would suggest that a diet containing a higher proportion of protein in the diet may have increased the probability of inducing hepatic neoplasms.

A particularly difficult aspect of the present study was refinement of laboratory techniques for long-term maintenance of English sole. Indeed, we had limited success maintaining disease-free test fish until we instituted prophylactic treatments with Furanace® and formalin during the first month of captivity. Alternatives to formalin as an effective treatment for ectoparasitic infestations simply do not exist and the options regarding effective alternate antibiotics were severely limited. Although both compounds are routinely used in fish culture, the possibility that these antibacterial and antiparasitic treatments may have contributed to lesion induction must be considered (Kimura et al. 1984; Swenberg et al. 1980). However, several factors argue against this being the case.

First, and foremost, experimentally induced hepatic lesions were detected almost exclusively among English sole exposed to the EHSE (both high and low doses) and BaP. Few lesion types were detected in controls, and at very low incidences. Second, the treatment concentration of Furanace® used was about five times lower than the lowest concentration shown by Kimura et al. (1984) to induce chromatophore hyperplasia or neoplasms in the croaker (*Nibea mitsukurii*). In addition, while Furanace® has been shown to induce hepatic neoplasms in ICR mice by subcutaneous injection or gastric ingestion (Kimura et al. 1984), no evidence of similarly induced hepatic neoplasia has been reported in fish. Kimura et al. (1984) observed a 4 to 13 times greater incidence of Furanace®-induced hepatic tumors in male versus female mice, and no such gender influence was observed with any of the lesions reported here. Finally, while formaldehyde is considered carcinogenic to the nasal epithelium in inhalation studies in mice and rats (Report on the Consensus Workshop on Formaldehyde 1984), it is considered a weak mutagen in most microbial mutagenicity tests (Auerbach et al. 1977) and other in vitro mutagenicity tests and is not mutagenic in any in vivo mutagenicity test (Report on the Consensus Workshop on Formaldehyde 1984). The consensus is that the cytotoxicity of formaldehyde and subsequent compensatory proliferative responses of the nasal epithelium in inhalation studies may contribute to the carcinogenicity of formaldehyde, and as such acts primarily as a promoter in the genesis of squamous carcinomas arising from the nasal epithelium.

There is no convincing experimental evidence that formaldehyde has primary toxic or carcinogenic effects at body sites distant from the site of exposure (Report on the Consensus Workshop on Formaldehyde 1984). Since formalin treatment was employed for parasitic infestation only once (for 1 h) prior to the start of the 18-mo test, the probability of it exerting such a cytotoxic or promotional effect is exceedingly low. Clearly, however, there is a need to identify other, less controversial chemotherapeutics for use in future studies of environmental carcinogenesis with wild fish species such as English sole.

In summary, the results of the present investigation represent an important step in establishing a direct link between exposure to sediment-associated chemical contaminants, principally aromatic compounds, and hepatic lesions, including neoplasms, in English sole. Although it required up to 18 mo to induce presumptive preneoplastic basophilic foci in the laboratory, this is one of only a very few models of hepatocarcinogenesis for which there are strong epizootiological data in the same free-living species implicating environmental contaminants. Heretofore, one of the major drawbacks associated with use of existing fish and rodent models has been the inability to relate laboratory results to field observations. This is not the case with the English sole model, and hence it would be particularly suited for laboratory studies delineating the processes and mechanisms comprising the multistep pathway of tumorigenesis in fish and mammals.

Acknowledgements

This project has been funded in part with funds from the Department of Health and Human Services under Interagency Agreement No. Y01-CP-40507. The contents of this publication do not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government. We thank L. D. Rhodes, L. L. Johnson, D. R. Bunnel, A. J. Friedman, D. G. Burrows, R. G. Bogar, J. A. Werner, J. C. Drury, K. L. Tilbury, O. Maynes, D. L. Del Beccaro, C. A. Wigren, R. W. Pearce, and M. Nishimoto for processing and analysis of samples, P. A. Robisch and P. Emry for preparation and fractionation of sediment extracts, and M. L. Baumgaertner and J. C. Cesarone for injection and maintenance of experimental fish. Appreciation is also given to S. Perry for typing.

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Physiological Responses to Challenge Tests in Six Stocks of Coho Salmon (*Oncorhynchus kisutch*)

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McGeer, J. C., L. Baranyi, and G. K. Iwama. 1991. Physiological responses to challenge tests in six stocks of coho salmon (*Oncorhynchus kisutch*). Can. J. Fish. Aquat. Sci. 48: 1761–1771.

Coho salmon (*Oncorhynchus kisutch*) from six hatcheries were reared in a common facility and then subjected to six different challenge tests. Results indicated that there are differences in the response to stressful challenges among stocks of coho salmon. The challenge tests were exposure to salt water (30 ‰), high pH (9.4 and 10.0), low pH (3.55, 3.65, 3.75, and 4.1), thermal increase (1°C·h^{-1}), disease (bacterial kidney disease), and handling (30-s netting and emersion). The measured responses were changes in plasma sodium and chloride ion concentrations for the saline and pH challenges, critical thermal maximum in the thermal tolerance test, mortalities in the disease challenge, and plasma glucose alterations in the handling challenge. The Chehalis River stock was most successful in tolerating salt water but showed the largest plasma ion decrease in acidic waters. The stock from Eagle River had the lowest plasma glucose increase during handling challenges. In the disease challenge the Tenderfoot Creek and Eagle River stocks had high mortalities but the Capilano River stock had the lowest mortality. No stock differences were found during thermal tolerance and high pH challenges. An assessment of overall stock performance across challenges showed that each stock had a unique response profile.

Des saumons coho (*Oncorhynchus kisutch*) provenant de six piscicultures ont été élevés dans un milieu commun et ensuite soumis à six types d'épreuves provoquantes. Les résultats indiquent que les réponses aux épreuves de stress diffèrent parmi les souches de saumon coho. Les épreuves étaient eau salée (30 ‰), pH élevé (9,4 et 10,0), pH bas (3,55, 3,65, 3,75 et 4,1) augmentation thermal (1°C·h^{-1}), maladie (maladie bactérienne du rein) et manipulation (capture aux filet et exposition à l'air pour 30 s). Les réactions mesurées étaient les changements du plasma en sodium et chlorure pour les test de salté et pH, température critique maximum dans l'augmentation thermal, mortalité dans l'épreuve de maladie et les changements de glucose du plasma pour les testes de manipulation. La souche de la rivière Chehalis tolerait mieux l'eau salée mais démontrait la plus grande diminution d'ion du plasma dans les eaux acides. La souche de la rivière Eagle avait le moins d'accroissement de glucose du plasma dans les épreuves de manipulation. Les mortalités étaient élevés pour les souches du ruisseaux Tenderfoot et de la rivière Eagle mais bas pour la souche provenant de la rivière Capilano dans l'épreuve de maladie. Il n'y avait aucune différence entre les souches pour les épreuves de tolérance thermal et pH élevé. L'évaluation de la performance des souches à travers les épreuves démontrent que chaque souche avait un profil de réponse unique.

Received October 31, 1990
Accepted April 5, 1991
(JA782)

Reçu le 31 octobre 1990
Accepté le 5 avril 1991

Species of Pacific salmon exist as a series of distinct subpopulations (stocks) that are reproductively isolated from each other (Horrall 1981). This isolation, when combined with habitat differences, has the potential to produce environmental adaptations that stocks preserve in their genotypes (Ricker 1972). Previous studies have shown that fish from similar environments have more in common with each other than those from different environments (Hjort and Schreck 1981; Wilmot and Burger 1985; Gharrett et al. 1987). Genetic variation among stocks can be demonstrated biochemically or from other phenotypic differences.

Genetic differences among coho salmon (*Oncorhynchus kisutch*) stocks on the west coast of North America have been demonstrated by electrophoretic separation of isoenzymes from polymorphic loci. An isoenzyme comparison of coho salmon by Reisenbichler and Phelps (1987) suggested that while stocks from watersheds within a given region were distinct, they shared more similarities than those from different regions. A larger isoenzyme study of coho salmon from southern British Columbia concluded that there were genetic differences among fish from the coastal mainland, Vancouver Island, and the upper Fraser River (Wehrhahn and Powell 1987).

Genetic differences among stocks for meristic and behavioural traits, as well as for characteristics important for intensive fish culture, have been observed in fish reared in common environments. Taylor and McPhail (1985a, 1985b) documented differences in body conformation and swimming ability between coastal and inland stocks of coho salmon. Behavioural diversity between coho salmon populations was demonstrated by Roseneau and McPhail (1987) and Swain and Holtby (1989). There is evidence, furthermore, that growth and survival vary among stocks of coho salmon from north, central, and southern British Columbia (B. Swift, University of British Columbia, Vancouver, B.C., pers. comm). Challenges with pathogens, such as *Renibacterium salmoninarum*, the causative agent for Bacterial Kidney Disease (BKD) (Suzumoto et al. 1977), and the parasite *Ceratomyxa shasta* (Envirocon Ltd. and E. V. S. Consultants Ltd. 1983; Ching and Parker 1989), demonstrated that stocks can respond differently.

Challenge tests that show differences between groups of fish can be used to assess the condition of fish (or fish populations) and to show the effects of environmental stress on fish (Wedemeyer and McLeay 1981; Beitinger and McCauley 1990). In this way, standardized challenge tests are a potential tool for monitoring health in fish culture and in environmental impact studies. Factors such as genetic makeup can affect the responses to challenge tests (Schreck 1981; Wedemeyer et al. 1984). Thus, such tests may be used to compare the tolerance limits or performance capacities among groups of fish. Our studies were designed to compare the performance capacity and tolerance limits of six different stocks of coho salmon from southern British Columbia by subjecting them to a variety of challenge tests. To illustrate differences among coho salmon stocks, selected physiological responses of juvenile fish from each stock were compared after exposure to salt water, low pH, high pH, temperature increase, a pathogen, and handling. The experiments were also used to assess the feasibility of using challenge tests to discern possible differences between groups of fish. The challenges were chosen from the suggestions of Wedemeyer and McLeay (1981) in their discussion of measuring the tolerance of fish to stressors using standardized challenge tests.

Materials and Methods

Approximately 2000 coho salmon were obtained from each of six Canadian Department of Fisheries and Oceans Salmonid Enhancement Project hatcheries and kept at the Aquaculture Unit of the Department of Animal Science, University of British Columbia. Stocks from the Capilano River (stock A), Chehalis River (stock B), Chilliwack River (stock C), Quinsam River (stock E), and Tenderfoot Creek (stock F) hatcheries were collected as eyed eggs in March 1988. The sixth stock (stock D) came from Eagle River Hatchery in June 1988 as 3.7-g fry. All fish collected were offspring of late-run adults, native to the watershed where the hatchery was located.

Fish were reared in dechlorinated Vancouver City tap water (temperature 4–15°C; hardness 4 $mg \cdot L^{-1}$ as $CaCO_3$; pH 5.8–6.2). All stocks were maintained in separate 150-L tanks with similar feeding rates, water flow, and management. Fish were fed a commercial salmon feed (Ewos ST40 and ST42). Estimates of individual weight from monthly samplings were used to develop growth curve projections (Iwama and Tautz 1981; Iwama 1982) in order to maintain feeding rates at satiation.

As the fish grew and loading densities increased, each stock was divided into two rearing groups. Stocks from rearing group 1 were maintained in the 150-L oval tanks, while stocks from rearing group 2 were kept in three equally split 750-L tanks. Natural photoperiod was approximated for both groups except for the period from October 1988 to May 1989 when a 12-h light and 12-h dark regime was used on all stocks in rearing group 1. The tank loading densities, water flow, and management were similar among stocks in each group but differed between groups.

At various times, fish from each stock were subjected to challenge tests that involved simultaneous exposure of fish from all stocks to a stressor (see Table 1 for details). The 24-h saltwater challenge test was conducted following procedures outlined by Blackburn and Clarke (1987). The disease challenge used a 0.1-mL inoculum of *Renibacterium salmoninarum* using methods described by Iwama (1980). The low pH challenge tests used a concentrated drip of HCl to decrease water pH to the desired level. The high pH challenges used NaOH to raise pH levels. In high pH challenges 1 and 2, the pH was increased to 7.5 for 24 h prior to the start of the challenge, at which time it was elevated to 9.40 and maintained for 72 h (high pH challenge 1) or 144 h (high pH challenge 2). The third pH challenge had a rapid increase to pH 9.3 at the start of the challenge followed by a gradual increase over the next 12 h to pH 10.00; pH 10 conditions were maintained for a further 12 h. The thermal tolerance challenge involved increasing the temperature by $1°C \cdot h^{-1}$ as outlined by McLeay and Gordon (1978). The handling challenges were 30-s netting and aerial emersion stresses as described by Barton et al. (1986) with sampling at 0, 1 (or 1.5), 3, 6, 9, 12, and 18 h. Weight (wt) and length (l) data collected during trials and sample weighings were used to calculate condition factors ($KF = 100 \cdot wt \cdot l^{-3}$, wt in grams and l in centimetres). Weight and length data were collected in December 1988 and January, February, and May 1989.

Unless otherwise noted, the following general procedures were carried out for all tests. The fish were starved for 12–24 h, nonselectivity netted from rearing tanks, fully anaesthetized, weighed, measured for length, and then transferred to a 48-L challenge box. This box was made of black perspex and divided into compartments so that fish from each stock could be housed and sampled separately but maintained in common water. Normal rearing water was supplied to the box at a flow-through rate of $4\ L \cdot min^{-1}$. Fish were challenged after a 24-h acclimation period. At the end of a trial, blood was collected from the caudal vasculature of killed fish ($NaHCO_3$-buffered tricaine methanesulfonate at $300\ mg \cdot L^{-1}$ or a blow to the head). After hematocrit (percent packed cell volume or %PCV) was determined, the plasma was saved and stored at −80°C for later determinations of plasma sodium ion concentration ($[Na^+]_{pl}$) and plasma chloride ion concentration ($[Cl^-]_{pl}$). In some trials, $[Cl^-]_{pl}$ was not measured. Control data were collected for each test by running a parallel trial that used all of the preparatory and sampling procedures but not the environmental change of the challenge.

For the saltwater, disease, thermal tolerance, and handling challenges the general procedures were modified. The saltwater challenges used a water-recirculation rather than a flow-through system. Water was aerated and temperature maintained (13°C) in saltwater challenge 1. The second saltwater challenge had controls and two challenge conditions. The first challenge condition was a 4°C increase in water temperature, while the other combined this temperature increase with saltwater exposure. The disease challenge used a control group, pooled from the six stocks, and each of the seven groups (six stocks and the one control) was housed in a 38-L aquarium. The acclimation period before inoculation was 42 d. Mortalities were collected daily and examined for signs of BKD (Iwama 1980). Beginning at 90 d after inoculation and continuing for 14 d, a daily stressor of netting the fish in each tank was applied. The thermal tolerance test did not use controls and the data collection consisted of noting the temperature at which fish either lost equilibrium or died (critical thermal maximum or CTM). In that test, water was recirculated at $3\ L \cdot min^{-1}$ and fresh water was added at a rate of $0.85\ L \cdot min^{-1}$. The handling challenges used a larger challenge box (150 L) and plasma was analyzed for glucose rather than plasma ion concentrations. There was no acclimation period and the 0-h measurement served as a control. As part of the second and third handling challenges, subsamples of fish were morphologically indexed for their stage of smol-

TABLE 1. Summary of challenges used in the comparison of six stocks of coho salmon juveniles (NA = not available).

Challenge test	Date (mo/yr)	Trial length	No. of fish per stock	Group used	Temp. (°C)	Environment change	Avg. fish weight (g)
Saltwater 1	10/88	24 h	15	1	13	30‰	9.5
Saltwater 2	12/88	24 h	9	1	7–11	30‰ + temp.[b]	16.1
Disease	10/88	5 mo	35–44	1	—[a]	BKD	NA
Low pH 1	12/88	24 h	9	1	7	pH 3.55	16.6
Low pH 2	05/89	20 h	6	1	9	pH 3.65	24.6
Low pH 3	05/89	20 h	6	2	9	pH 3.65	17.4
Low pH 4	05/89	24 h	6	1	9	pH 3.75	23.5
Low pH 5	05/89	24 h	6	2	9	pH 4.10	17.0
High pH 1	11/88	72 h	6	1	9	pH 9.40	12.8
High pH 2	11/88	144 h	6	1	9	pH 9.40	12.8
High pH 3	02/89	24 h	6	1	4	pH 10.0	17.6
Thermal	03/89	22 h	8	1	6	1°C/h	NA
Handling 1	06/89	18 h	6–7[c]	1	10	Net and emerse	26.7
Handling 2	06/89	18 h	7–8[c]	2	10	Net and emerse	25.2
Handling 3	06/89	18 h	5–8[c]	1	10	Net and emerse	26.8

[a]Temperature ranged from 4 to 13°C.
[b]4°C increase in water temperature.
[c]Number of fish per stock per sample time.

tification using the four-level index (1 for parr to 4 for smolt) of Gorbman et al. (1982).

Plasma ion concentrations were determined using flame photometry for $[Na^+]_{pl}$ (model 410, Corning Instruments) and coulometric titration (Chloridometer, Haakebuchler Instruments Ltd.) for $[Cl^-]_{pl}$. Plasma glucose concentrations were determined colourimetrically by the glucose oxidase method (Sigma Diagnostics, St. Louis, MO, procedure No. 510) using a Shimadzu UV-VIS spectrophotometer UV-160 (Shimadzu Corp. Kyoto, Japan) at a wavelength of 450 nm.

Means for measured features for each stock under control and challenge conditions were tested for significant differences by ANOVA using a six (stocks) by two (water treatments) factorial experiment with completely random design. A one-way ANOVA was used to compare stocks in the temperature and handling trials as well as the condition factor data. Where appropriate, stock means were compared using Tukey's HSD test. In the handling challenges, Dunnett's test was used to compare each 0-h mean with subsequent sample means. Disease challenge mortality to survival ratios were compared by chi-square analysis. The error level for all statistical tests was 0.05.

The response of stocks was also compared across the challenges. For challenges that showed differences among stocks, each stock was ranked according to the magnitude of change in one of the measured parameters. The magnitude of change rankings used the difference between the control mean and the mean post-challenge for either $[Na^+]_{pl}$ or plasma glucose concentration, depending on the challenge. Ranking within the disease challenge was done using percent cumulative mortality. Comparisons of rank among the replicates for each type of challenge and among all challenges were conducted by ANOVA. The relative response of each stock to each type of challenge was also quantified and compared. The relative response was quantified by assigning a relative numeric value from 1 to 6. This relative response score evaluated the overall response of a stock (cumulative mortality or hematocrit changes and either $[Na^+]_{pl}$ and $[Cl^-]_{pl}$ changes or plasma glucose changes) to each type of challenge. An assigned relative response score of 1 equalled no change from resting or control values and a score of 6 indicated an acute and life-threatening condition.

Results and Discussion

Saltwater Challenges

The fish used in the saltwater challenge of October 1988 lost weight. Hematocrit values also decreased during the 48 h (24-h acclimation plus 24-h challenge) in the challenge box. The average weight loss was less in control fish (mean loss of 0.7 g) than in challenged fish (mean loss of 1.2 g). The overall mean for hematocrit in control conditions was higher (52 %PCV) than in challenged fish (46 %PCV). Under challenge conditions, stocks B, C, and D had significantly lower hematocrit values than under control conditions.

Plasma ion concentrations were significantly increased for all stocks when they were exposed to salt water, but the magnitude of the increase differed between stocks. Under control conditions, there were no differences in plasma ions among stocks. The overall mean was 139 $mEq \cdot L^{-1}$ for $[Na^+]_{pl}$ and 132 $mEq \cdot L^{-1}$ for $[Cl^-]_{pl}$. In salt water, stock B showed an increased $[Na^+]_{pl}$, but it was significantly less than the increases other stocks experienced (Fig. 1). Stocks A and C had intermediate $[Na^+]_{pl}$ increases, while stocks D, E, and F showed the highest increases. The $[Cl^-]_{pl}$ were also significantly increased under challenge conditions but there were fewer significant differences among stocks. Stocks A, B, and C had smaller $[Cl^-]_{pl}$ increases than stocks E, F, and G (Table 2).

The second saltwater challenges was a combined temperature increase and saline challenge. In the salt–temperature treatment, mortalities occurred in all stocks. Mortality rate was highest for stocks D and C (100% mortality), lowest for stocks A and F (30% mortality), and intermediate for stocks B and E (60 and 80% mortality, respectively). Hematocrit was elevated from control conditions in the temperature increase only challenge for stocks A, C, D, and F. After the temperature increase and saltwater challenge, survivors from stocks A and E had

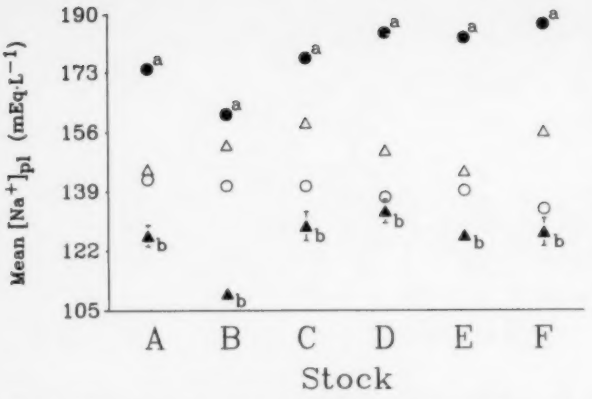

FIG. 1. Mean (±1 SE) $[Na^+]_{pl}$ for six stocks of coho salmon from southern British Columbia during saltwater challenge 1 (open and solid circles, $n = 15$) and low pH challenge 1 (open and solid triangles, $n = 9$). Open circles and triangles designate control means, while solid circles and triangles indicate challenged means. The letter "a" indicates a challenge mean significantly greater ($P < 0.05$) than the control mean for that stock. The letter "b" indicates a challenge mean significantly less ($P < 0.05$) than the control mean for that stock.

hematocrits that were significantly lower than control values. Mean values for $[Na^+]_{pl}$ were lowest for controls, intermediate in the temperature increase only conditions, and highest for fish exposed to temperature increase and salt water. Stocks A and C were the only groups that showed significant $[Na^+]_{pl}$ increases and stock D recorded the smallest increase when controls were compared with the temperature increase treatment (Table 2).

The first saltwater challenge showed that some stocks were able to tolerate saline conditions, although life history information would not have predicted that result. While fish from stock B were most able to regulate $[Na^+]_{pl}$ and could be considered as functional smolts, fish from stocks A and C may also have been competent, as they were most successful in maintaining $[Cl^-]_{pl}$. Fish that will survive and grow in salt water should be able to regulate $[Na^+]_{pl}$ to 170 mEq·L^{-1} or less within 24 h during a saltwater challenge (Clarke and Blackburn 1977; Clarke 1982; Iwata et al. 1982; Johnson and Heifetz 1988).

The results of the two saltwater challenges, when combined, illustrate the cumulative effects of stress. The cumulative nature of stress has been shown by Flos et al. (1988) with grading and transport of rainbow trout (*Oncorhynchus mykiss*, formerly *Salmo gairdneri*) and also by Barton et al. (1986) with multiple handling treatments of chinook salmon (*Oncorhynchus tshawytscha*). There is a bioenergic cost associated with maintaining homeostasis under stressful conditions, and as stresses accumulate, animals are less able to compensate (Barton and Schreck 1987b). Stock A was sensitive to the temperature increase but had fewer mortalities than other stocks under the dual stress of temperature increase and saline water. This same stock had intermediate ion changes in the first saltwater challenge. Stock D, which had large plasma ion changes in the first saltwater challenge, was relatively unaffected by temperature increase alone, but had 100%

TABLE 2. Means by stock and treatment for $[Cl^-]_{pl}$ and $[Na^+]_{pl}$ in six stocks of coho salmon from southern British Columbia after exposure to 30‰ salt water (saltwater challenge 1) or to 30‰ salt water combined with a 4°C temperature increase (saltwater challenge 2). Standard errors are in parentheses; NA = not available, as this treatment caused 100% mortality.

	Stock					
	A	B	C	D	E	F
Saltwater challenge 1						
$[Cl^-]_{pl}$ (mEq·L^{-1})						
Control	131 (1.3)	129 (1.5)	137 (4.1)	133 (2.4)	131 (2.0)	132 (2.5)
Saltwater	160[a] (2.2)	166[a] (4.7)	162[a] (5.9)	180[a] (5.3)	173[a] (5.0)	169[a] (3.7)
$[Na^+]_{pl}$ (mEq·L^{-1}) (see Fig. 1)						
Saltwater challenge 2						
$[Cl^-]_{pl}$ (mEq·L^{-1})						
Control	133 (1.6)	132 (1.7)	138 (3.0)	126 (1.3)	125 (2.4)	138 (3.0)
4°C increase	149 (6.0)	144 (2.4)	141 (13.0)	137 (2.0)	128 (1.1)	140 (2.3)
Salt + 4°C increase	189[b] (7.7)	170[b] (5.5)	NA	NA	196[b] (6.5)	176[b] (5.5)
$[Na^+]_{pl}$ (mEq·L^{-1})						
Control	145 (0.9)	152 (1.9)	159 (2.2)	151 (1.4)	145 (1.7)	156 (1.6)
4°C increase	173[a] (5.1)	164 (1.4)	176[a] (6.7)	152 (1.2)	149 (1.3)	169 (6.0)
Salt + 4°C increase	195[b] (5.2)	189[b] (5.4)	NA	NA	208[b] (9.0)	184[a] (3.5)

[a]Challenge mean significantly different from control mean.
[b]Significantly different from control and temperature increase treatment.

mortality under conditions of salt water and increased temperature.

Low pH Challenges

In most of the low pH challenge tests, all stocks experienced higher hematocrit values and significant reductions in plasma ion concentrations (Table 3; Fig. 1). During low pH challenge 1, there were no significant differences in fish weight. Water conditions (control or low pH) had a significant effect on average hematocrit value, with a mean for control fish of 47 %PCV and for challenged fish of 59 %PCV. There were no differences among stocks within each of the treatments. There was a significant decrease in $[Na^+]_{pl}$ for all stocks in low pH water. Stock B had much lower $[Na^+]_{pl}$ than all other stocks (Fig. 1). Mean $[Cl^-]_{pl}$ for all stocks were significantly reduced in acid waters and stock B exhibited the largest reductions (Table 3).

Results of the low pH experiments of May 1989 were generally consistent with those of the December challenge. In low pH challenge 2, all fish had increased hematocrit values and reduced $[Na^+]_{pl}$ (Table 3). There were no differences in hematocrit among stocks under control (mean PCV of 44%) or challenge conditions (mean of 61 %PCV). In low pH water, stock E was the only one that did not show a significant increase in hematocrit when compared with its control. All stocks had similar $[Na^+]_{pl}$ under control and challenge conditions. When mean $[Na^+]_{pl}$ for stocks in the low pH treatment were ranked, stock B had the lowest value.

In low pH challenge 3, hematocrit increased in all stocks. The mean %PCV was 41 for control fish and 59 for challenged fish. The low pH conditions also produced significantly reduced $[Na^+]_{pl}$ for all stocks except E (Table 3). Stock B displayed the greatest $[Na^+]_{pl}$ decline. This was the only low pH challenge that had mortalities. Mortalities were 50% for stock C and 18% for stock F.

The fourth and fifth low pH challenges had intermediate pH value treatments. At pH 3.75 (low pH challenge 4), there were significant increases in hematocrit in fish from stocks B, C, and D and no change in $[Na^+]_{pl}$ for all stocks (Table 3). At pH 4.1 (low pH challenge 5), there was an increase in hematocrit in fish of stocks A, B, C, and F and a slight but significant decrease in $[Na^+]_{pl}$ for stocks A, B, and C (Table 3).

The five low pH challenges demonstrated that exposure to acidic conditions caused significant physiological disturbances

TABLE 3. Means by stock and treatment for $[Cl^-]_{pl}$ and $[Na^+]_{pl}$ in six stocks of coho salmon from southern British Columbia after exposure to pH 3.55, 3.65, 3.65, 3.75, and 4.10 (low pH challenges 1, 2, 3, 4, and 5, respectively). Standard errors are in parentheses.

	Stock					
	A	B	C	D	E	F
Low pH challenge 1						
$[Cl^-]_{pl}$ ($mEq \cdot L^{-1}$)						
Control	133 (1.6)	132 (1.7)	138 (3.0)	126 (1.3)	125 (2.4)	138 (3.0)
Low pH	101[a] (4.9)	82[a] (6.1)	101[a] (5.8)	105[a] (5.7)	94[a] (1.5)	90[a] (3.4)
$[Na^+]_{pl}$ ($mEq \cdot L^{-1}$) (see Fig. 1)						
Low pH challenge 2						
$[Na^+]_{pl}$ ($mEq \cdot L^{-1}$)						
Control	145 (2.6)	136 (3.2)	139 (2.7)	138 (1.4)	138 (2.5)	139 (1.4)
Low pH	133 (2.7)	122 (1.7)	132 (3.7)	128 (4.9)	130 (3.8)	130 (5.7)
Low pH challenge 3						
$[Na^+]_{pl}$ ($mEq \cdot L^{-1}$)						
Control	152 (1.4)	151 (1.2)	151 (0.7)	146 (1.0)	148 (1.2)	147 (1.5)
Low pH	130[a] (2.0)	117[a] (3.1)	122[a] (10.4)	132[a] (2.9)	141 (2.5)	121[a] (7.1)
Low pH challenge 4						
$[Na^+]_{pl}$ ($mEq \cdot L^{-1}$)						
Control	145 (2.6)	136 (3.2)	139 (2.7)	138 (1.4)	138 (2.5)	139 (1.4)
Low PH	140 (2.2)	136 (2.7)	136 (2.7)	135 (2.6)	137 (2.0)	145 (1.7)
Low pH challenge 5						
$[Na^+]_{pl}$ ($mEq \cdot L^{-1}$)						
Control	152 (1.4)	151 (1.2)	151 (0.7)	146 (1.0)	148 (1.2)	147 (1.5)
Low pH	141[a] (0.6)	143[a] (1.1)	142[a] (1.3)	142 (1.0)	145 (2.5)	141 (1.9)

[a]Challenge mean significantly different from control mean.

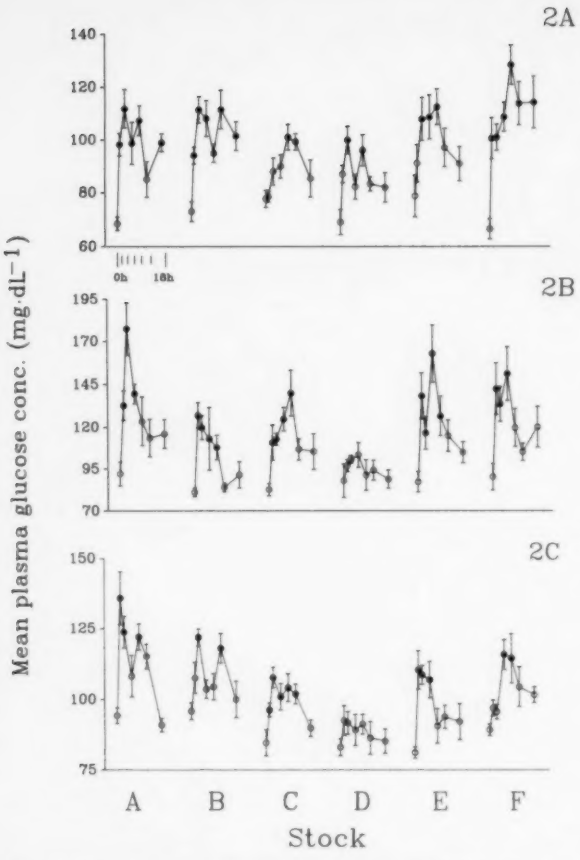

FIG. 2. Mean (±1 SE) plasma glucose concentration of six stocks of coho salmon from southern British Columbia sampled at 0, 1 (or 1.5), 3, 6, 9, 12, and 18 h after the 30-s netting and emersion of (A) handling challenge 1, (B) handling challenge 2, and (C) handling challenge 3 (n = 5–8). Open circles are not statistically different ($P > 0.05$) from initial (0 h) values for that stock.

(hematocrit value increase and ion loss) that were most severe for stock B and least severe for stock E. In all trials that showed significant physiological changes, fish in stock B consistently had the largest reductions in $[Na^+]_{pl}$ and $[Cl^-]_{pl}$. Stock E usually displayed smaller losses in $[Na^+]_{pl}$ than other stocks. Schom (1986), using data from Atlantic salmon (*Salmo salar*), and Swarts et al. (1978), using data from brook trout (*Salvelinus fontinalis*), suggested that the ability to survive acidic conditions is better in fish from native waters of a low pH. Robinson et al. (1976) also concluded that acid tolerance of brook trout had a genetic component. The available records do not indicate anything unique about the water quality, in this regard, in the watersheds or hatcheries from which stocks B and E originated.

Handling Challenges

In the first of the three handling challenges, there were no mean hematocrit or plasma glucose concentration differences among stocks at the 0-h control samples. The overall mean for initial hematocrit was 48 %PCV. At subsequent sampling times, all stocks showed no change in hematocrit compared with 0-h control values but had significant increases in plasma glucose concentration (Fig. 2A). Relative to other stocks, the magnitude of plasma glucose increase was smallest for stocks D and C and greatest for stocks F and A.

The second handling challenge also had no mean hematocrit or plasma glucose differences among stocks at the 0-h control sample. At all sampling times for all stocks except C at 18 h, no change in mean hematocrit from initial values (0 h) was observed. Mean initial hematocrit was 51 %PCV. All stocks showed a significant rise in mean plasma glucose concentration from initial values at one or more of the sampling times except stock D (Fig. 2B). Stock A had the largest increase in plasma glucose concentration. The smoltification index data showed that all stocks were at a similar stage with an overall mean index value of 3.97.

In the third handling challenge, mean hematocrit among stocks was different before the challenge. Stock D hematocrit was lowest at 46 %PCV, while the other stocks ranged from 48 to 52 %PCV. During the 18-h postchallenge period, there was no significant change in hematocrit from initial values for any stocks. Mean resting (0 h) plasma glucose concentrations also did not vary between stocks (Fig. 2C). Stock A fish had the largest increase in plasma glucose concentration, while those of stock D did not show a significant increase at any of the sampling times. For all stocks, there were no significant differences between 0- and 18-h samples. The mean smoltification index of 3.4 for stock A was significantly lower than those for other stocks (3.90–4.00).

The three handling challenges demonstrated that there were significant differences among stocks in stress-induced responses in plasma glucose. Plasma glucose is considered to be a reliable physiological indicator of stress (Mazeaud et al. 1977; Wedemeyer and McLeay 1981; Wedemeyer et al. 1990). Stock D showed the smallest glucose response to the handling stress and stock A the largest. Variation between wild and hatchery stocks for stress-induced blood glucose concentration changes has been shown in rainbow trout (Wydoski et al. 1976; Casilla and Smith 1977; Woodward and Strange 1987), largemouth bass (*Micropterus salmoides*) (Williamson and Carmichael 1986), and chinook salmon (Barton et al. 1986). Refstie (1986) found that there was a genetic component to stress-induced blood glucose concentration changes among different groups of Atlantic salmon.

There were no differences among stocks in hematocrit response to the handling challenges. The lack of change in hematocrit values probably reflected the sampling times. The hematocrit changes observed by Casilla and Smith (1977) in response to a hooking stress were most dramatic in the first 20 min after the disturbance. Our first sample was not taken until 1 or 1.5 h after handling and, thus, may have missed any acute transitory change.

Disease Challenge

The disease challenge resulted in significant differences in mortality among the six stocks. There were no mortalities or signs of BKD in the control group. Stocks C, E, and F had intermediate levels of cumulative mortality (42, 50, and 61%, respectively). Cumulative mortality in stock A (36%) was significantly lower compared with all other stocks. Stocks B and D experienced mortality rates higher than the other stocks (83 and 81%). All dead fish displayed similar internal signs, characteristic of BKD (Iwama 1980).

Studies with rainbow trout in seawater (Refstie 1982) and Atlantic salmon in freshwater (Gjedrem and Aulstad 1974) have shown that resistance to *Vibrio anguillarum* can vary among stocks. A study with steelhead trout (*Oncorhynchus mykiss*), chinook, and coho salmon (Ching and Parker 1989) and one with chinook salmon (Envirocon Ltd. and E. V. S. Consultants Ltd. 1983) demonstrated that resistance to the parasite *Ceratomyxa shasta* also varied among stocks. Those studies showed that fish stocks naturally exposed to the pathogen in their migration routes survived better when challenged under experimental conditions. Suzumoto et al. (1977) linked differences in BKD resistance among three groups of coho salmon to different transferrin genotypes.

The BKD exposure experiment reported here was actually a combination of disease and mild physical disturbance but it is likely that the observed differences among stocks were due to the BKD and not the applied stressor. As stated in the review by Wedemeyer and Goodyear (1984), the stress response results in a readjustment of metabolic processes and, if the stress is chronic, the fish will be less able to defend themselves against disease. Maule et al. (1989), using chinook salmon, and Ellsaesser and Clem (1986), using channel catfish (*Ictalurus punctatus*), have shown that acute stress compromises immune system function and disease resistance. In this experiment, stock A, which showed the largest glucose increase in response to handling, also had the lowest mortality rate. Stock D showed the lowest glucose response to handling and a high mortality rate. The reasons for these observations are not known.

High pH Challenges

The three high pH challenges produced few significant changes in physiological constituents among stocks of fish. Hematocrit values were unaffected by the challenge conditions at 72 h (high pH challenge 1). The mean hematocrit was 45 %PCV for control fish and 46 %PCV for challenged fish. At 144 h (high pH challenge 2), hematocrit tended to be lower in challenged fish (mean of 37 %PCV) than in controls (mean of 42 %PCV). In the second challenge, decreases in hematocrit were only significant for stocks B and C. At 72 h, the mean $[Na^+]_{pl}$ was significantly decreased from control values in stocks B, C, and D, while only stock F had lower $[Na^+]_{pl}$ at 144 h (Table 4). Mean $[Cl^-]_{pl}$ was not altered significantly by the high pH exposure at either time except in stock F. At 72 h, stock F had a significantly higher $[Cl^-]_{pl}$ than its control. The third high pH challenge exposed fish to a higher pH for a shorter time. The alkaline conditions did not result in any hematocrit changes (mean of 43 %PCV for each treatment). Mean $[Na^+]_{pl}$ and $[Cl^-]_{pl}$ did not differ significantly among stocks or treatments except in stock A which had a significantly lower $[Na^+]_{pl}$ level after the high pH challenge (Table 4).

The high pH challenges did not result in any significant physiological changes within stocks or differences between stocks. The reasons for the absence of change under challenge conditions may include combinations of the sampling time, applied water pH, and the parameters measured. At pH values above 9.54 the inhibition of ammonia exchange is almost complete and ammonia levels will increase within a few hours (Wright and Wood 1985). Yesaki (1990) found that 0.41-kg rainbow trout showed significant $[Na^+]_{pl}$ decreases after approximately 24 and 48 h at pH 10.1. Therefore, while it was expected that significant changes would occur in plasma ion concentrations in high pH challenge 3, few significant changes occurred. Challenges at higher pH for a longer time and use of parameters such as plasma ammonia concentration may be needed to demonstrate physiological differences among stocks exposed to high pH.

Temperature Tolerance Challenge

The temperature tolerance challenge did not show differences in CTM among the stocks. All fish survived at temperatures up to 23°C but none survived at higher than 25.5°C. Stock C had the lowest CTM at 23.8°C and stock D the highest at 24.4°C.

The CTM for all stocks was within a narrow range, the upper limit of which was close to the lethal temperature reported for coho salmon (Piper et al. 1982). The CTM was also 0.7°C less than that reported for coho salmon acclimated to a similar rearing temperature (Becker and Genoway 1979). There is evidence for stock and genetic differences in CTM for other species of fish. For example, Fields et al. (1987) found that a Wisconsin stock of largemouth bass (*Micropterus salmoides*) had a significantly lower CTM than a Florida stock. Possible explanations of the similarities in CTM in our experiments may be the small difference in geographic and latitudinal ranges among the stocks involved as well as the similar habitat temperatures in the locations from which our stocks originated.

Overall Response to Challenges

The ranked response for each of the challenges showed consistent differences within the repeated challenges but not between challenges. Within the low pH challenges, stock B consistently showed the highest disturbance with a significantly higher mean rank than stocks C and E. The handling challenges also showed a consistent trend, where stock D had a mean rank which was significantly lower than stock A. Comparison of mean rank across challenges showed no significant differences. Mean rank ranged from 3.1 for stock E to 4.3 for stock B.

There were also no significant differences among the stocks in relative response scores. While the individual challenge tests results may reveal interesting and potentially important traits, the challenges should also be viewed in combination (Fig. 3). A profile of various measurements was used by Buckley et al. (1985) to assess the effects of environmental degradation by comparing stocks of largemouth bass. That concept was used to combine the results of challenging tests to show differences between stocks of fish. The data from this experiment showed that there was an interaction between relative response score and stock. Therefore, the performance of a stock varied with the challenge applied and each stock was unique in its overall response to the challenges.

Other Observations

Condition factor (KF) changed over time and there were differences in KF among stocks (Fig. 4). Stock D had significantly lower mean KF at all times measured. All stocks except C and F showed a significant decrease in KF over time. The decrease in KF occurred between January and February for stocks A and E and February and May for stocks B and D. The morphometric data confirmed reported differences in body conformation between coastal and inland stocks of fish. These condition factor results are similar to those of Taylor and McPhail (1985a). They demonstrated that inland stocks of coho salmon tended to be long and thin, while those from coastal locations

TABLE 4. Means by stock and treatment for $[Cl^-]_{pl}$ and $[Na^+]_{pl}$ in six stocks of coho salmon from southern British Columbia after exposure to pH 9.4, 9.4, and 10.0 (high pH challenges 1, 2, and 3, respectively). Standard errors are in parentheses.

	Stock					
	A	B	C	D	E	F
High pH challenge 1						
$[Cl^-]_{pl}$ (mEq·L^{-1})						
Control	124	122	122	128	124	122
	(1.8)	(0.9)	(1.9)	(1.8)	(2.2)	(1.5)
High pH	127	130	128	126	130	132[a]
	(2.3)	(1.9)	(1.1)	(2.0)	(5.3)	(2.4)
$[Na^+]_{pl}$ (mEq·L^{-1})						
Control	150	153	146	149	145	148
	(2.3)	(1.4)	(1.9)	(0.7)	(2.2)	(1.4)
High pH	148	140[a]	136[a]	139[a]	140	141
	(2.2)	(0.9)	(1.2)	(2.8)	(1.6)	(1.8)
High pH challenge 2						
$[Cl^-]_{pl}$ (mEq·L^{-1})						
Control	130	128	130	130	130	130
	(1.5)	(1.9)	(1.4)	(1.1)	(1.8)	(1.5)
High pH	131	130	132	129	129	127
	(1.3)	(0.5)	(1.3)	(1.6)	(1.2)	(1.5)
$[Na^+]_{pl}$ (mEq·L^{-1})						
Control	146	151	146	150	146	150
	(1.5)	(1.5)	(1.6)	(0.8)	(5.2)	(1.0)
High pH	137	144	149	151	140	139[a]
	(1.6)	(1.2)	(3.4)	(2.2)	(1.6)	(2.1)
High pH challenge 3						
$[Cl^-]_{pl}$ (mEq·L^{-1})						
Control	137	130	137	134	137	137
	(3.3)	(3.3)	(2.4)	(1.9)	(2.4)	(2.9)
High pH	144	133	133	122	131	138
	(1.6)	(2.3)	(3.6)	(1.7)	(1.3)	(2.1)
$[Na^+]_{pl}$ (mEq·L^{-1})						
Control	156	150	149	150	140	143
	(1.5)	(1.5)	(1.5)	(3.2)	(2.5)	(2.4)
High pH	136[a]	152	147	160	138	150
	(1.3)	(2.0)	(2.5)	(3.5)	(3.1)	(1.4)

[a]Challenge mean significantly different from control mean.

tended to be short and deep bodied, and concluded that these distinctions had a genetic basis. In our groups of fish, stock D was the only one from an inland source. Our results also demonstrate that there are body conformation changes over time. As salmon undergo the transition from parr to smolt, there are a series of morphological changes that occur, including a decrease in KF (Gorbman et al. 1982; Winans and Nishioka 1987). Barton et al. (1985) documented decreases in KF in coho salmon that were quite similar to the changes we observed in the coastal stocks.

Our results showed that the two rearing groups differed in both resting (control) and challenge conditions (see Table 1 for group use details). In unaltered water conditions (controls), group 1 fish had significantly higher hematocrits and lower $[Na^+]_{pl}$ than group 2 fish. When challenged by exposure to pH 3.65, stocks C and F in group 2 had mortalities, while those from group 1 did not. At the same pH, stock E fish of group 2 showed no reduction in $[Na^+]_{pl}$, while those in rearing group 1 did. The magnitude of the maximum mean plasma glucose elevation differed also between groups for most stocks. Plasma glucose concentrations in fish of stocks A, C, E, and F were much higher in group 2 than in group 1. Factors which can alter stress-induced glucose changes such as rearing temperature (Barton and Schreck 1987a), nutritional status (Barton et al. 1988), and stocking density (Congelton et al. 1984) may have caused these differences between groups 1 and 2; a definitive explanation, however, cannot be deduced from the data.

Ion concentrations in resting fish, especially $[Na^+]_{pl}$, changed significantly in the period from October to May. As rearing temperature decreased from 13 to 4°C, there were significant increases in $[Na^+]_{pl}$ and decreases in hematocrit. As the rearing temperature rose in May and June, these trends were reversed.

The blood sampling conducted before and during the three handling challenges showed a diurnal variation in resting plasma glucose. Variation in resting glucose concentrations has been shown by Congelton et al. (1984). In that study, levels were low at night and elevated during the day. Our data, however, contradict that pattern but are in agreement with Barton et al. (1986) who showed decreasing levels as the day progressed.

Discussion and Conclusions

Our experiments have demonstrated that certain stocks of coho salmon juveniles show distinctly different responses to

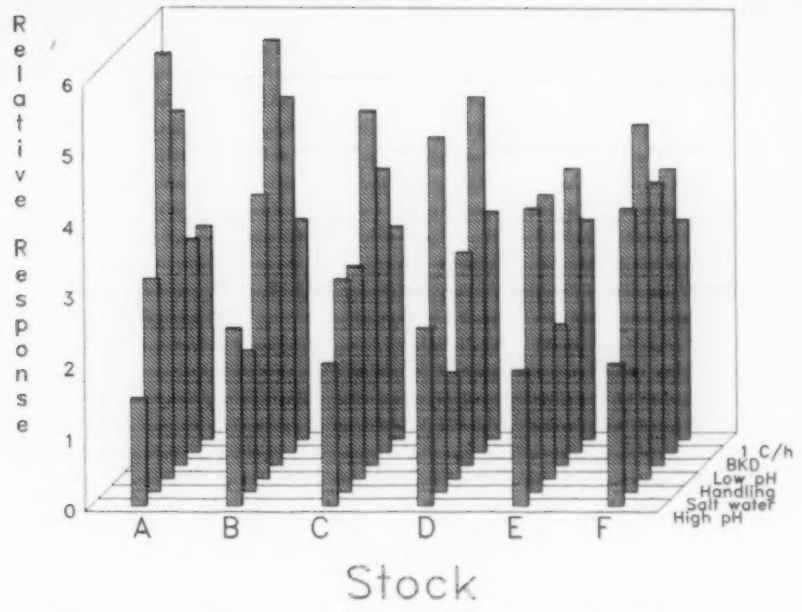

FIG. 3. Relative response profile of six stocks of coho salmon from southern British Columbia. Bars show the disturbance experienced by each stock (relative to other stocks) in the challenges. The score assigned to each stock for each type of challenge ranged from 1 (no effect) to 6 (largest disturbance).

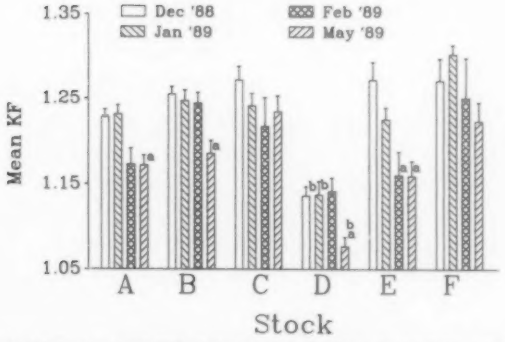

FIG. 4. Mean (±1 SE) KF of six stocks of coho salmon from southern British Columbia at four time periods. Bars with the letter "b" are significantly lower ($P < 0.05$) than other stocks at that sampling time, and the letter "a" denotes means significantly lower ($P < 0.05$) than December 1988 measurements for the same stock.

challenges. Stock B appeared to possess an unusual osmoregulatory ability, stock D displayed a low metabolic response (as measured by plasma glucose concentration) to handling, and stock A was the most resistant to BKD. The statistical analysis of the overall performance profile shows an interaction between stock and the response to different challenges and supports the hypothesis that stocks possess unique characteristics in their response to environmental changes. The study design limited environmental differences among stocks during rearing (from eyed egg onwards) and it is reasonable to suggest that the observed differences had a genetic basis. Further supporting evidence is offered by the replicated challenges which produced similar results when fish were raised under different housing and management conditions. Therefore, it is possible that there are genetic differences among stocks of coho salmon that result in significantly different responses to challenge tests. We recognize that the response to the challenges may have been influenced by innate differences among stocks in smoltification schedule or reaction to dechlorinated Vancouver water.

Challenge tests have a wide variety of application and purposes (reviewed by Wedemeyer et al. 1990; Beitinger and McCauley 1990) and they can be useful in selecting genotypes that are physiologically suited to tolerate stress (see Schreck 1981). The use of a variety of short-duration challenge tests to develop performance profiles of stocks of fish could make a valuable contribution to the existing base of knowledge in that regard. Results of this type are potentially useful for improving rearing and enhancement management, selecting stocks for transplantation or selection and breeding in commercial aquaculture, monitoring environmental degradation, and demonstrating the importance of management policies that include the maintenance of stocks. When the use of challenges and the development of performance profiles are contemplated, it is important to identify factors which may alter responses. For example, the influence of temperature, photoperiod, smoltification, stage of maturity, diurnal patterns, and rearing environment should be considered.

Acknowledgements

Funding was provided by the Natural Sciences and Engineering Research Council of Canada as an operating grant to G.K.I. and a postgraduate scholarship to J.C.M. Some of the work was also supported by the National Centers of Excellence as part of the Canadian Bacterial Diseases Network Research Program. Animals were donated by the Canadian Department of Fisheries and Oceans. The management and personnel of the six source hatcheries were helpful. The

assistance of D. Creelman, E. Teng, I. Bernatsky, and R. Creelman is also gratefully acknowledged.

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An Analytical Model of Acoustic Fish Reflection for Estimation of Maximum Dorsal Aspect Target Strength

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We develop a simple analytical model of the acoustic reflectivity of fish targets based on empirical observations in field and laboratory settings. It enables unbiased estimation of maximum dorsal aspect target strength in cases where individual fish are observed a minimum of three times in successive pings of a survey transect. The model results are consistent with observations of example fish targets of two demersal species. It promises improved estimates of target strength from field measurements.

Nous avons mis au point un modèle analytique simple de la réflectance acoustique des cibles à partir d'observations empiriques de poissons effectuées sur le terrain et en laboratoire. Ce modèle permet d'obtenir une estimation non biaisée de l'effectif maximum de la cible sur la face dorsale dans les cas où les individus sont observés un minimum de trois fois en impulsions successives sur un transect. Les résultats du modèle montrent une cohérence avec les observations d'exemples de cibles de deux espèces démersales. Ce modèle promet d'améliorer les estimations de l'effectif des cibles à partir des mesures effectuées sur le terrain.

Received September 5, 1990
Accepted March 13, 1991
(JA714)

Reçu le 5 septembre 1990
Accepté le 13 mars 1991

Application of acoustic methods to estimate fish abundance requires techniques for calculating the in situ acoustic size, the target strength (TS), of individual fish. Both echo integration and echo counting methodologies require an estimate of the TS of individual sonified targets. In the former, an appropriate average is needed to scale the total accumulated squared voltage or signal power from acoustic reflections during survey transects (Foote 1987; MacLennan 1990). In the latter, TS is needed for compiling population size composition. Acoustic estimates of fish biomass depend on the relation between TS and physical body size of the fish. This relation is known to be very sensitive to the density of targets as well as their physical and biological state. As a result, a field method of establishing TS is preferable to a priori estimates.

Effective sizing of fish in their natural surroundings offers advantages for the study of fish populations (Boudreau and Dickie 1989). For example, the degree of local or mesoscale aggregation of fish appears to be related to body size, so size information is important for understanding changes in catchability or availability (Boudreau 1992; Crecco and Overholtz 1990). But most importantly, a strong correlation between populations sized acoustically and those sampled by fishing gear provides the best method currently known for calibrating field abundance estimation methodologies, thus enabling acoustic estimates of abundance to be placed on an absolute basis.

Given a calibrated acoustic system, three steps are needed to relate acoustic size to biological fish size: (1) measurement of echo level (EL) and position of the target in the beam, (2) relation of the observed EL to a standard TS, and (3) estimation of fish body size from the standard TS. A key consideration is the meaning of "standard TS." Since it must be related unambiguously to fish length, we define it as the TS of a fish observed in some preferred aspect, taken here to be the dorsal aspect where TS attains its maximum value.

There is little controversy about step 1. Much recent development of echo sounder systems for fish survey has been directed to improved measurement of position (Foote et al. 1986) and improved algorithms for resolving individual targets (Dickie et al. 1983). Step 3 presents more difficulty. Even under controlled conditions the relation between fish size and maximum dorsal aspect TS varies between targets. The relation depends on species (Love 1977) and degree of activity (Clay and Heist 1984), and probably on body condition (Ona 1990). These are sources of uncertainty that cannot yet be resolved by acoustic information alone. There is, nevertheless, compelling evidence that, within practically definable classes of biological targets, there exists a linear relationship between TS and log (length) (Foote 1979). The two regression parameters can be experimentally derived within practically useful limits.

It is step 2 that presents the greatest difficulty. There is significant variance about the relationship between measured EL and the standard TS which has been ignored or addressed by inadequate statistical methods. This variance has prevented full utilization of the substantial data sets potentially available from acoustic survey. This paper attempts to overcome the deficiency by employing a simple model of the acoustic properties of fish reflectance that can be treated analytically to extract information on fish size and aspect from acoustic data.

We outline an analytical model derived from empirical observations to estimate the dorsal aspect TS of individually

observed fish. The model can be fitted by field observations where a fish target is sonified at several positions in the beam. Model results are compared with examples from field data, collected in this case using the dual-beam ECOLOG I. The result can be seen to generate estimates of standard (maximum dorsal) TS with an accuracy not hitherto available. Although we restrict the present development to the dual-beam system, the principle is applicable to data gathered by either dual-beam or split-beam acoustic techniques, so long as individual targets can be observed from several perspectives in successive pings.

The merit of the model is that it shows how a simple analytical form chosen for the TS function provides a framework for extrapolating a sequence of equally spaced EL observations to the maximum dorsal TS. Information hitherto discarded may be used to guide the data analysis to this end. Refinements such as better modeling of the TS function, treatment of noise, rejection of data cases not satisfying our assumptions of fish behaviour, etc., are deliberately omitted so as not to detract from the key elements of the development.

Effects of Aspect on TS and Data Requirements for the Model

Love (1977) reported changes in observed TS when a fish is viewed from different positions on the unit sphere constructed around it. In species from 14 different families representing seven orders, TS varied in a systematic manner when referenced to the longitudinal axis of the fish. His finding that TS reaches a maximum when viewed from the dorsal aspect, that it falls off in regular fashion when viewed from various aspect angles along the plane of bilateral symmetry (pitch or tilt), and is little affected by roll is in agreement with observations of Nakken and Olsen (1977).

More precise definition of the TS function with aspect has become important with the development of dual-beam and split-beam echo sounder systems that provide measures of target position with respect to the acoustic axis. The geometry of observations for single fish was studied by Foote (1980), who presented data on preserved specimens of the North Sea pollack (*Pollachius pollachius*) and saithe (*P. virens*) (Foote 1985). Observations on living Atlantic cod (*Gadus morhua*) by Dickie et al. (1984) and by Dickie and Boudreau (1987) showed that aspect angle and position in the beam combine to produce a systematic perspective effect on EL, giving the prospect of simultaneous corrections for aspect and position on the calculations of fish TS.

In the model that follows, we envision an echo sounder that measures the angular position of a target from the central axis of the sonified cone, defined by the two angles θ and φ (Fig. 1). In the model for the dual-beam system, we deal only with off-axis angular displacement, since information on azimuthal angle φ is not given by the dual-beam. Target range from the transducer, R, is given by the time after transmit. Throughout, we assume that the data are collected using a time-varied gain (TVG) to correct in standard fashion ($40 \log R + 2aR$) for effects of range spreading and absorption (a). We assume the adoption of echo sounder frequencies, pulse lengths, and other equipment settings to be suited to the target sizes and ranges observed. The discussion of results is independent of particular settings. We note also that the particular system used here to collect field data was calibrated using the copper calibration sphere technique (Foote et al. 1987).

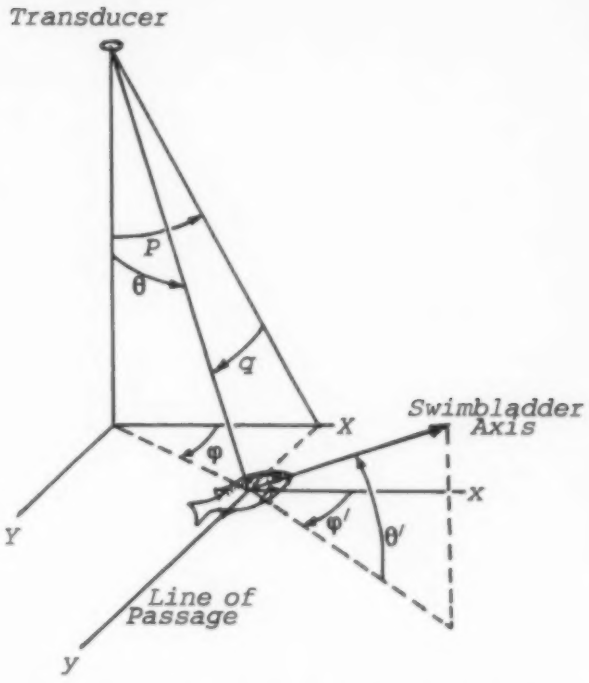


FIG. 1. Coordinates of fish location and orientation in the sonified acoustic cone.

In our model, we consider the case where data are collected by a vertically oriented transducer towed at constant speed along a linear transect. If the ratio of ping rate to towing speed is sufficiently high, individual targets beyond a given distance from the transducer are detected not only in several of the vertical time samples within a ping, but horizontally several times over successive pings. We assume that the data processing techniques have scanned the water column information for a target "envelope" within individual pings and have selected a peak, or other standard value, for further processing. We also assume a preliminary method, such as constant range, for tentatively identifying a succession of peaks from successive pings as belonging to a single target or "fish envelope" (Dickie and Boudreau 1987).

The sequence of observations of a single fish over successive pings may be considered as equally spaced points along a line of passage that appears as a chord drawn through the horizontal cross-section of the sonified cone (Fig. 1). A sequence of such observations mapped into a suitable EL coordinate system is defined here as a trajectory. Our model uses such a trajectory to estimate the dorsal aspect TS of the observed target. A limitation of the model is that the maximum dorsal TS is indeterminate if the observed target has been detected fewer than three times (successive pings) along the chord. That is, the acoustic system needs to have settings that yield a sonified cone wide enough to permit multiple observations of individual fish within the field of view while restricting the individual sample volumes so that the probability of multiple targets is as low as practicable.

In analyzing acoustic data for abundance or density, it is essential to determine the volume of water sonified by the

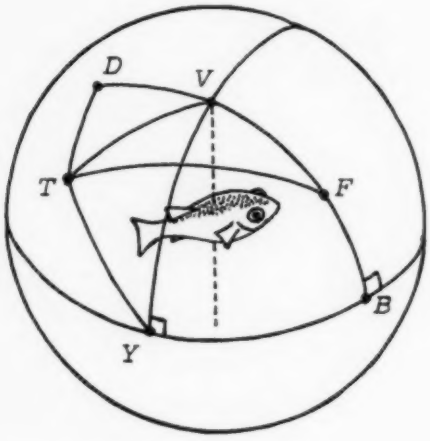
equipment. This is specified in different ways for split-beam and dual-beam systems. Alternative methods available for the dual-beam are treated below. For purposes of the analytical model, we assume that the volume can be specified in terms of the system parameters and that the angle of the field of view is small, of the order of a few degrees. Our parameterization is in terms of EL's detected by the two transducers of a dual-beam system and permits direct comparison of the model with actual dual-beam data. Application to split-beam systems requires parallel parameterization of the comparable split-beam trajectory.

Three-Parameter Swimbladder Model

For convenience, we ascribe all significant variation in TS to changes in swim bladder aspect and characterize the model in terms of the swimbladder geometry. Other body parts also reflect energy (Sun et al. 1985), but the requirement for the model is only that the empirical data reflect the principal aspect dependence of TS and that this is measured by EL and position information. Angular coordinates underlying the model are defined in Fig. 1 and 2. We consider the swimbladder reflector to be a slender, approximately linear object with symmetry about three perpendicular planes and with a reference longitudinal axis found, on average, in a near horizontal direction. The sagittal plane of bilateral symmetry contains the swimbladder axis and is assumed to be always effectively vertical. A second plane of symmetry (approximately horizontal) intersects the sagittal plane along the swimbladder axis. As explained below, this latter assumed symmetry is only a matter of convenience. The third plane contains the transverse cross-section of the swimbladder.

The TS functions evaluated in directions normal to these three planes are the three parameters defining the model. These observed target strengths, normal to the transverse (F_0), normal to the horizontal (F_1), and normal to the sagittal (F_2), are seen in the near frontal, near dorsal, and lateral aspects, respectively. In accordance with observations of actual fish, F_0 and F_1 are the overall minimum and maximum TS, respectively, along directions in the plane of bilateral symmetry. The near dorsal direction along which the maximum dorsal aspect TS, F_1, is measured will be called simply the dorsal direction.

We recognize that appreciable departures from the above assumed symmetries are observed in nature (Foote 1985). The assumptions have been made to allow development of a simple analytical form by which maximum dorsal aspect TS can be extracted from the EL trajectory. In principle the method could be extended to asymmetric and correspondingly more compli-



V: *Vertical Direction*

F: *Swimbladder Axis*

D: *Dorsal Direction*

Y: *y-axis*

$\overline{DVFB}$: *Sagittal Plane*

$\overline{YB}$: *Horizontal Plane*

$\overline{DF} = \overline{YV} = \overline{BV} = \frac{\pi}{2}$

Fish Location	*Swimbladder Orientation*	*Aspect*
$\theta = \overline{VT}$	$\theta' = \overline{BF}$	$\Theta = \overline{DT}$
$\varphi = \angle YVT - \frac{\pi}{2}$	$\varphi' = \frac{\pi}{2} - \angle YVF$	$\Phi = \pi - \angle TDF$
$p = \angle VYT$		$\alpha = \overline{FT}$
$q = \overline{YT} - \frac{\pi}{2}$		$\gamma = \angle VFT$

FIG. 2. Geometry of TS function on the unit sphere centred on a fish target for comparison with definitions of Foote (1985).

cated analytic forms, but the essential modeling requirements for a swimbladder TS function seen in a small field of view are still accuracy and smooth analytical behaviour in the neighbourhood of the dorsal direction. It is immaterial, for example, that we fail to model accurately the dorsal–ventral asymmetry observed in swimbladders (Foote 1985). In particular, the near horizontal plane of symmetry is built into the model for simplicity and convenience only.

The coordinates in Fig. 1 describing the position and orientation of the swimbladder axis within the field of view are adopted from Foote (1980). The fish appears to pass through the field of view in the horizontal X–Y plane along a straight line parallel to the Y-axis. The x- and y-axes are centred on the swimbladder and are parallel to the X- and Y-axes, respectively. Angle p denotes the offset of the line of passage from the vertical sonar axis, and q denotes the (increasing) apparent displacement angle of the swimbladder as seen from the transducer. The alternate variables θ and φ, describing the location of the fish, and the variables θ' and φ', describing the orientation of the swimbladder longitudinal axis as in Foote's paper, are all defined in Fig. 1. We depart from Foote's usage only in that the field coordinates necessarily describe orientation of the swimbladder axis (or other structures that reflect sound) rather than the physical fish axis. Angle θ' is called the tilt angle, often quoted as having a mean value of $-4.4°$ (Olsen 1971) when it is referenced to the horizontal. The swimbladder position and orientation are determined by specifying the four coordinates p, q, θ', and φ'. If an effectively immobile fish passes through the field of view on any one of a set of simple parallel translations or chords, the coordinates p, θ', and φ' on each translation will remain constant; q is the only coordinate that changes.

Application of spherical trigonometry to this system is made easier when the relationships are redrawn on the unit sphere centred on the fish as in Fig. 2. Foote's apparent scatterer orientation angles Θ and Φ, again referenced to the swimbladder axis rather than the fish axis, are indicated in the figure as well as our preferred choices α and γ. In words, α is the polar angle from the swimbladder axis to the transducer, while γ is the corresponding azimuthal angle measured from the plane of bilateral symmetry.

We model the TS function in the general form

$$(1)\quad \mathrm{TS} = F_0 \cos^2 \alpha + (F_1 \cos^2 \gamma + F_2 \sin^2 \gamma) \sin^2 \alpha.$$

An example of the beam pattern given by this model is shown in Fig. 3 with $F_1 - F_0 > F_1 - F_2$ and $(F_1 - F_0):(F_1 - F_2) :: 9:2$. Sections through the three principal planes show simple lobed structure similar to the smoothed envelopes that can be drawn around polar plots of observed fish TS (Love 1977). We emphasize that eq. (1) should be regarded as a prototypical model approximating the largest scale features of a swimbladder TS function in the neighbourhood of the dorsal direction. Other more familiar three-dimensional surfaces might serve just as well in approximating these features, but we find that they seem to have intractable analytical properties which when repaired force us back to eq. (1).

Observed or computed TS functions are usually rich in lobe-like structures. Eq. (1) undertakes only to represent the one lobe containing the maximum dorsal aspect TS. Consequently, it is important that the observations which enter the analysis are made with some certainty on this lobe, i.e. in the near dorsal direction. This requirement is usually satisfied for well-behaved fish passing through a sufficiently narrow acoustic beam. In quantitative and uncomplicated terms, "sufficiently narrow" roughly means the beam angular diameter should be less than the lobe width (acoustic wavelength/effective swimbladder length) in the sagittal plane. In practical terms for the implementation of this model, the passage of a badly behaved fish is likely to yield data that will be rejected as unusable by the model. As pointed out later, there are data cases that must be so rejected.

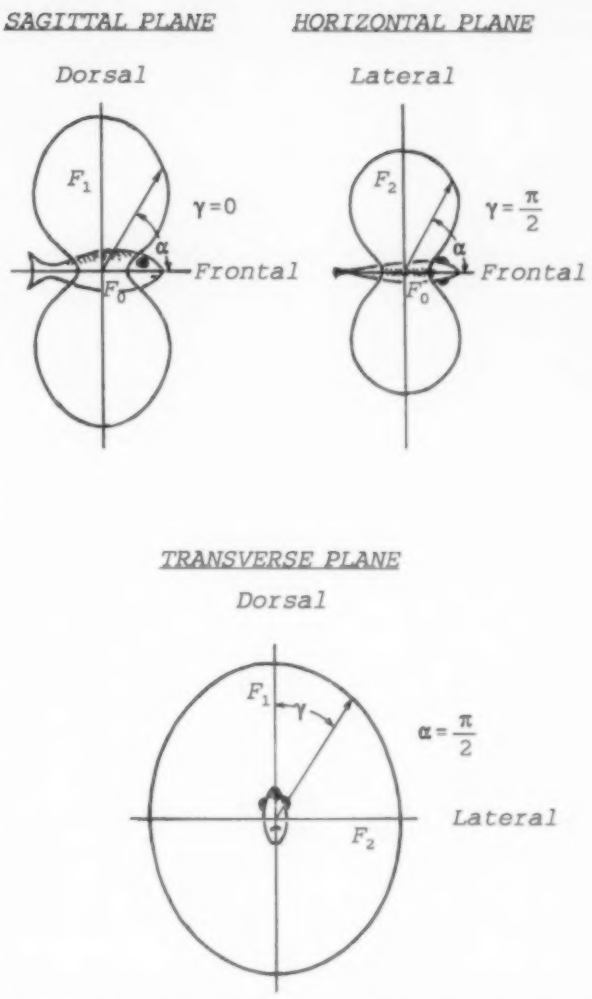


FIG. 3. Polar plots of modeled TS functions: sections through the three major planes from which the fish can be viewed.

In this connection the parameter F_0 must not be interpreted literally as the near frontal aspect TS of the real swimbladder. In the context of approximating a real swimbladder TS function with eq. (1), F_0 should be considered a parameter related to the curvature of the real TS function in the sagittal plane near the dorsal direction. It turns out that F_0 is indeterminate in the context of fitting our model to data. However, the method is capable of estimating an upper bound on F_0 which is likely to have a value appreciably less than the real frontal aspect TS, since real TS functions seem to be more directional than that modeled by eq. (1).

It is possible to rewrite this expression in terms of the four coordinates p, q, θ', and φ', examine its dependence on the changing coordinate q, and then determine whether the model parameters can be deduced from observed trajectories of EL or the equivalent TS. In the following section, this is carried out for a somewhat simpler version of the model than that represented by eq. (1).

Axially Symmetric Target

A model with symmetry about the swimbladder longitudinal axis is obtained by letting the normally minor effects of roll angles be represented by $F_2 = F_1$, thereby reducing eq. (1) to the two-parameter axially symmetric model

$$(2) \quad \mathrm{TS} = F_0 + (F_1 - F_0) \sin^2 \alpha.$$

For gadoids, such axial symmetry is observed to be a reasonably good approximation in the neighbourhood of the dorsal direction where our interest resides. Hence, eq. (2) will be taken as the swimbladder model in the remainder of this paper.

Our objective is to extract the maximum dorsal aspect TS, F_1, given a sufficient description of the trajectory observed when a fish passes through the field of view along a chord.

The fact that F_1 is determinable at least under some conditions follows from the argument that (1) full axial symmetry implies that the TS is F_1 in any direction lying in the transverse plane, (2) relative to the fish, the transducer moves in a straight line which must intersect this plane unless the plane is parallel to the line; barring for the moment this special case, (3) the event of transducer–transverse plane intersection, whether it happens inside or outside the field of view, gives the measurement of F_1, and (4) if enough of the trajectory lies in the field of view or can be inferred by an extrapolation from the visible part, then a simple search on the trajectory for maximum TS finds the intersection point giving F_1. The only target property needed for this construction is symmetry in the transverse plane containing the maximum TS. Additional analytical properties of the model, eq. (2), can be used to carry out the extrapolation.

In the normally expected case where there is not full axial symmetry, the above procedure is modified by limiting its application to that portion of a trajectory consistent with an axially symmetric model fitted to the real swimbladder in the neighbourhood of the dorsal direction. This works because all is determined by the behaviour of the TS function in the neighbourhood of the dorsal direction. In practice, it is this local behaviour, inferred from observations, that will be analyzed for F_1, thus effectively extrapolating and searching the trajectory.

In place of the changing coordinate q, we introduce the coordinate

$$(3) \quad t = \tan q = \sin p \tan \varphi$$

which has the useful property of being linearly proportional to a coordinate that measures distance along the line of passage. At the point of closest approach to the transducer axis, $t = 0$. Working with the relations defined in Fig. 2, we find that

$$(4) \quad \sin^2 \alpha = (A' + B't + C't^2)/(1+t^2)$$

where A', B', and C' are functions of p, θ', and φ'. The explicit forms of these coefficients are not needed in this development. We note only that they have fixed values for any particular passage of a fish through the field of view. Inserting eq. (4) into eq. (2) gives

$$(5) \quad \mathrm{TS} = (A + Bt + (A+C)t^2)/(1+t^2)$$

where the unprimed coefficients A, B, and C are linearly related to A', B', and C' with coefficients linear in F_0 and F_1. Again we note only that A, B, and C have fixed values for any particular passage.

As argued above, there is nearly always some value of t for which the maximum TS is attained and this value is F_1. Analytically, it is given by

$$(6) \quad F_1 = (2A + C + \sqrt{B^2 + C^2})/2.$$

The positive root in eq. (6) is always correct and the expression even handles the special case when the line of passage is parallel to the plane of symmetry (the maximum then occurs in the limit $|t| \to \infty$). Eq. (6) can be confirmed by a direct but laborious computation using the explicit forms of A', B', and C'. If the trajectory implied by eq. (5) is observed at least in part and the unknown coefficients A, B, and C are somehow extracted from the known elements of the trajectory, then our objective is achieved by evaluating eq. (6).

Application

Dual-Beam Parameterization

The ECOLOG I dual-beam acoustic system configuration is described elsewhere (Dickie et al. 1983). It has two coaxial nested transducers, one with a narrow beam angle (half-power points at about 3°) and the other with a beam angle wide enough (half-power points at about 26°) to be considered flat within the field of the narrow beam. The sound pulse is transmitted on the narrow and received on both wide and narrow. The basic transducer equations may then be written as

$$(7) \quad E_N^2 = k_N b_N^2 (\theta,\varphi)\, \sigma$$

$$(8) \quad E_W^2 = k_W b_N (\theta,\varphi)\, b_W (\theta,\varphi)\, \sigma$$

where E_N^2 is the power received on the narrow transducer, E_W^2 is the power received on the wide transducer, $k_{W,N}$ are the system calibration constants, b_N (θ, φ) is the beam pattern of the narrow beam, b_W (θ, φ) is the beam pattern of the wide beam where θ and φ are the spherical coordinates of beam position defined in Fig. 1, and σ is the backscattering cross-section which is constant with angle of view for spherical scatterers but varying for asymmetrical targets.

The volume of water sonified by a dual-beam system may be defined in two different ways. In one, a fixed threshold angle off the axis may be set by reference to a given ratio of power levels measured on the two transducers. Alternatively, the minimum acceptable detection threshold power level, E_d^2, may be used in conjunction with known beam pattern functions to define the volume. Since diameter of the field of sonification is dependent on the EL of the observed target, an a priori threshold corresponding to the smallest targets of interest limits the volume that is sampled for all larger targets. In data collections underlying this paper, we have, accordingly, set a detection threshold power level, E_d^2 on the narrow beam transducer such that a 10-dB signal to noise ratio is maintained for the smallest targets of interest. The beam pattern of the narrow beam is then used to establish the volume sampled for each target size, σ_o. That is, for a fixed value of $\sigma = \sigma_o$, the region in the θ, φ space that satisfies the equation

$$E_d^2 = E_N^2 = k_N b_N^2(\theta,\varphi)\, \sigma_o$$

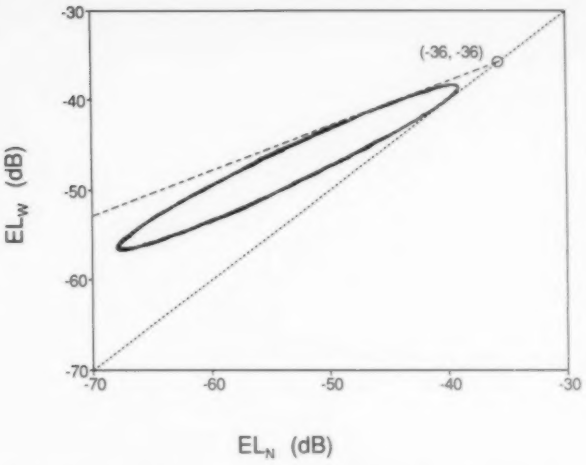


FIG. 4. Elliptical trajectory in the (EL_N,EL_W) coordinate system, based on parameters for a −36.0-dB fish. For illustrative purposes, no detection threshold is imposed and the narrow beam half-angle is exaggerated by widening to 30°. The dotted line is the 1:1 line and the broken line is the construction line with slope 0.5 tangent to the ellipse.

defines an angular "field of view" for the target. The development of the model assumes that there is a finite field of view, and while its exact definition in relation to target size is not critical, the maximum angle for large fish objects should be of the order of the spread of the half-power points of the narrow transducer.

In acoustic analyses, data are expressed in decibel form and the system constants are absorbed in the measurements. Thus we may write for the corresponding EL's on the two transducers

$$\text{(9a)}\quad EL_N = 10 \log (E_N^2/k_N) = 20 \log b_N(\theta,\varphi) + 10 \log \sigma$$

$$\text{(9b)}\quad EL_W = 10 \log (E_W^2/k_W) = 10 \log b_N(\theta,\varphi) + 10 \log \sigma.$$

The TS estimate derived from the dual-beam system, which we will define here as u, may then be calculated as

$$\text{(10)}\quad u = TS = 10 \log \sigma = 2\,EL_W - EL_N.$$

The only assumption made in deriving the above result, beyond the validity of the transducer equations, is that the wide beam pattern $b_W(\theta, \varphi) = 1$ over the field of view established by the narrow beam (Traynor and Ehrenberg 1979). Any calculation of TS at a given position in the beam is independent of the particular beam pattern of the narrow beam transducer and applies to any target within the field of view.

In the dual-beam system, eq. (10) may be used directly to construct a nomogram to determine TS from EL measurements. Using an idealized beam pattern, to be introduced below, and a simulated TS function, we generate a trajectory which is plotted in (EL_N, EL_W) coordinates in Fig. 4. It can be seen from eq. (10) that a line with slope of 0.5 through any point on the trajectory intersects the 1:1 line ($EL_W = EL_N$) at the point (TS, TS). The locus of EL pairs for a spherical scatterer, such as a copper calibration sphere, lies along a straight line with slope 0.5; hence, TS may be estimated without bias from any point along it (Dickie and Boudreau 1987). However, where there is significant effect of aspect on TS, such as with the fish target simulated in Fig. 4, the succession of (EL_N, EL_W) points gives rise to a family of curves. In such a case the ideal dual-beam eq. (10) cannot be used to estimate on-axis or dorsal aspect TS from off-axis observations. The model developed here specifically addresses this problem for the dual-beam parameters.

General Form of the Acoustic Trajectory

To provide heuristic support for the model, it is informative to examine the trajectory of EL pairs implied by eq. (5) in conjunction with an assumed idealized transducer beam pattern. Suppose the fish is observed using a system having a narrow beam pattern given in decibel form by

$$\text{(11)}\quad -10 \log b_N(\theta) = s \sin^2(\theta) = s\,(\sin^2 p + t^2)/(1 + t^2)$$

where s is positive and could be regarded as a known system parameter depending on the acoustic wavelength and transducer size. The beam pattern, whether or not idealized, is henceforth assumed to be axially symmetric, i.e. a function of θ only, as in eq. (11). The form of eq. (11) is applicable to real transducers for small angles only, where terms of fourth and higher degree in θ (or t and p) can be ignored. For example, with an on-axis osculating fit of eq. (11) to the beam pattern of a circular piston transducer, the absolute error is ±0.18 dB at the half-power (3 dB) point. The narrow and wide EL for the dual-beam transducer are then, respectively,

$$\text{(12a)}\quad EL_N = [A - 2s \sin^2 p + Bt + (A + C - 2s)\,t^2]/(1 + t^2)$$

$$\text{(12b)}\quad EL_W = [A - s \sin^2 p + Bt + (A + C - s)\,t^2]/(1 + t^2).$$

This pair of equations defines an ellipse in the (EL_N, EL_W) coordinate system.

An objection may be raised against the improbable form of the idealized beam pattern embodied in eq. (11) if extended to large angles. The form chosen is one of a number of possibilities that are valid within a small field of view. This particular choice combines algebraically very easily with eq. (5) to produce an ellipse. Other possible beam patterns, including that of the real system, would produce different (and generally intractable) trajectory shapes. This is not a problem for the axially symmetric model, since analysis of all shapes must yield the same F_1 for any passage of a given fish through the field of view.

Eq. (12) was used to plot the ellipse in Fig. 4. In general, EL pairs lie above the 1:1 line, possibly touching it at only one point which would represent the point of passage of the fish through the axis of the sonified cone. The synthetic elliptical trajectory of Fig. 4 is based on properties of a −36.0-dB fish observed in the dorsal direction plotted with no field of view limits and is a simple and concrete example as a reference in developing the model. The point $EL_N = -44.70$, $EL_W = -40.35$ is the point corresponding to maximum TS.

As pointed out by Dickie and Boudreau (1987), in the dual-beam system, a TS-independent measure of the off-axis distance of the target is given by the ratio of the wide to the narrow received voltages. In terms of eq. (9) or (12), this measure is calculated from the difference between the wide and narrow EL, which we will define as v. Using eq. (9), we may thus write the equation

$$\text{(13)}\quad v = EL_W - EL_N = -10 \log b_N(\theta,\varphi).$$

For the split-beam system, a comparable measure of distance off-axis is afforded by phase difference measurements in the X and Y dimensions.

Given information on position in the sonified beam, the EL_N in eq. (9a) or (12a) for the dual-beam may be used in conjunction with eq. (13) to define another coordinate system (v, EL_N) in which a trajectory can be plotted and the effects of aspect angle directly displayed. In this coordinate system the sequence of observations of EL_N at various points in the beam, which represent observations made in successive pings in a survey transect, is plotted against v, giving the observed trajectory. Comparable trajectories are obtained using EL measured by either the split-beam system or by the narrow beam of the dual-beam system. However, as noted above, the abscissa of the coordinate system, representing the distance off-axis, is measured differently in the two systems.

A (v,u) coordinate system for the TS function may be defined for the two systems. For the dual-beam it is calculated by inserting information from eq. (9) or, for the modeled swimbladder, eq. (12) in eq. (10). For the split-beam, an equivalent TS is calculated by adjusting EL for the beam pattern using information on phase shifts among beams. It is these estimates of TS that provide the information we use as a basis for studying the effects of aspect on echoes from real fish targets at different positions in the beam. Plotting them as ordinates (u) on abscissae (v) representing the distance off-axis provides visual appreciation of the fit of data to the model. The three coordinate systems (EL_N, EL_W), (v,EL_N), and (v,u), being connected by affine transformations, preserve parallel straight lines and transform ellipses into ellipses and (of significance below) parabolas into parabolas. Thus the ellipse of Fig. 4 maps into an ellipse in the other two coordinate systems. We study a simplified version of these plots in more detail below (Fig. 5) in connection with the fitting of field data.

For sufficiently small values of the tilt angle, i.e. if $|\theta'| < |p|$, it is possible for the ellipse to collapse to a straight line. This happens in the situation where the maximum TS occurs when the target is closest to the transducer axis, i.e. at $t = 0$. In the coordinate system of Fig. 4, it can be shown that the slope of such a straight-line ellipse must lie between 0.5 and 1. Correspondingly, slopes of averaged trajectory data seem to lie roughly between these limits. The empirical fittings of Dickie and Boudreau (1987), showing an average slope of about 0.75 but with a considerable range of values about it, point to this phenomenon in field data. The large range of values correspond with observations by Olsen (1971) and Buerkle (1983) on aspect angles in the field that the conditions for a precise, straight-line trajectory from fish occur only rarely in the field.

Small Angle Approximation: Parabolic Trajectories

A practical simplification, leading to more insights, follows from the small angle approximation applied to eq. (12). Dropping terms of third and higher degree in t and p, we obtain

$$\text{(14a)} \quad EL_N = A - 2s\,p^2 + Bt + (C - 2s)\,t^2$$

$$\text{(14b)} \quad EL_W = A - s\,p^2 + Bt + (C - s)\,t^2$$

which define a parabola in any of the three coordinate systems. The small angle approximation leading to parabolic trajectories is appropriate (irrespective of beam pattern) for describing portions of trajectories lying in the field of view, since the latter is typically only a few degrees wide.

Figure 5 shows the parabolic approximation of an elliptical trajectory for a −36.0-dB fish in all three coordinate systems. Figure 5A is analogous to Fig. 4 restricted to points above a detection threshold of −45.0 dB. The TS function with fish

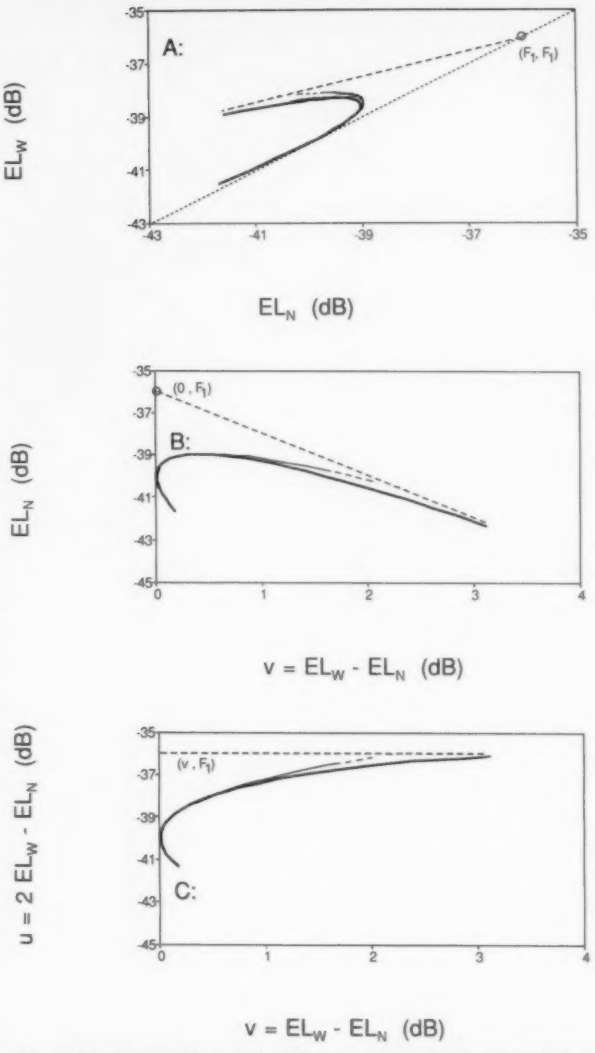


FIG. 5. Trajectories for a −36-dB fish target. Both elliptical and parabolic (small angle approximation) trajectories are plotted. The detection threshold is set at −42 dB. The heavy solid line is the ellipse; the narrow solid line with broken extension is its approximating parabola. The broken line tangent to the ellipse is the construction line for estimating F_1. (A) Trajectories in (EL_N, EL_W) coordinates; (B) trajectories in (v, EL_N) coordinates; (C) trajectories in (v, u) coordinates.

aspect used in generating Fig. 5 was taken from Foote's (1985) data using −36.0 dB as the maximum dorsal aspect TS, F_1, and fish tilt angle equal to zero. The narrow beam pattern was modeled in simple numerical fashion similar to eq. (11).

In Fig. 5A, it is apparent from the broken construction line with slope of 0.5 drawn tangent to the fitted parabolic form and intersecting the 1:1 line at −36.0 dB that for this fish the maximum TS occurred when it was at the edge of the sonified cone, corresponding to the −4.4° difference between the fish and the swimbladder axes. Figure 5B shows the same EL trajectory displayed in the (v, EL_N) coordinate system derived from eq. (9a) and (13). Its parabolic form illustrates the large systematic variation of the EL with position in the beam.

Data from both dual- and split-beam systems can be represented in (v, u) coordinates for the parabola of TS against distance off-axis. Figure 5C represents it for the dual-beam by plotting u from eq. (10) against v from eq. (13). This figure illustrates the change in TS with position in the beam with the effect of the beam pattern removed. Here, it is more apparent that the maximum TS estimated from the parabola occurs at the margin of the sonified cone. The spread of the arms of the parabola is diagnostic of situations where the TS maximum occurs appreciably off the axis of the transducer. The deviation, $-4.4°$, between the acoustic axis of the fish, F_1, and of the transducer results in an on-axis observation of TS that is over 3.5 dB less than the actual maximum dorsal value.

One might infer from the foregoing that, as a route to the determination of F_1, it is indirect as well as inefficient to construct the unobserved parts of any trajectory. There are many different possible beam patterns, all of which are acceptable in a dual-beam system; there are also many different possible trajectories all giving the same F_1. The parabola impartially approximates all possible trajectories, since all share the same visible portion in the field of view. The method proposed for actually carrying out the computation is based on working directly with the approximating parabola given by eq. (14) that fits this visible portion. If then the coefficients A, B, and C can be found from the parabola, eq. (6) gives F_1.

Fitting the Parabolic Trajectory: Equal Interval Measurements

Before proceeding with the computational details, we reiterate the steps developed thus far for finding the maximum dorsal TS: (1) assemble data for the EL diagram or observed trajectory, (2) find the approximating parabola, (3) analyze the parabola for the coefficients A, B, and C, and (4) compute F_1 from eq. (6).

In practice, we must work with a set of imprecise and noisy EL measurements, presumably belonging to the same fish. It is reasonable to bypass questions about the unseen parts of the trajectory and work directly with the approximating parabola. The computation thus depends on a sequence of EL and position measurements on a fish, such as the trajectory variously displayed in Fig. 5A, 5B, or 5C where the fish is assumed to be relatively immobile, acoustically modeled by eq. (2), and in uniform motion through the field of view. The points with coordinates (F_1, F_1), $(0, F_1)$, and (v, F_1) are indicated on Fig. 5.

Suppose that n time-ordered measurements (n successive pings) are made of the wide and narrow EL: $(EL_W)_i$ and $(EL_N)_i$, respectively, with $i = 1,\ldots,n$. Let t_i be the corresponding initially unknown values of the coordinate t. If the measurements are made at equal time intervals, as is usual in echo sounding devices, then the t_i are also separated by equal intervals Δt. This is a key element in the analysis and comprises hitherto unused information about EL observations of tracked targets. Under some conditions, Δt can be determined independent of the EL measurements, but in the following, it is initially regarded as an unknown.

Using the model to recognize a parabolic TS trajectory resulting from the effects of perspective, we then form the TS and off-transducer-axis distance combinations, u_i and v_i of eq. (10) and (13), to obtain from eq. (14)

$$u_i = A + Bt_i + Ct_i^2 \tag{15}$$

$$v_i = s(p^2 + t_i^2). \tag{16}$$

Eq. (15) and (16) open a way for solving for A, B, and C: we first solve or find the best values of t_i and p in eq. (16) and then subsequently use the values of t_i in eq. (15) to obtain A, B, and C.

Noting that $t_i = t_1 + (i-1)\,\Delta t$, we can rewrite eq. (16) as

$$v_i/s = p^2 + t_1^2 + 2(i-1)\,t_1\,\Delta t + (i-1)^2\,(\Delta t)^2 \tag{17}$$

which, while nonlinear in the unknowns p, Δt, and t_1, is of the form $v_i/s = a + b(i-1) + c(i-1)^2$ and therefore intrinsically linear in the independent unknowns a, b, and c. A linear regression of v_i/s on $(i-1)$ and $(i-1)^2$ gives best values of a, b, and c in terms of which we obtain the estimates

$$\Delta t = \sqrt{c} \tag{18}$$

$$t_i = [b + 2(i-1)c]/2\sqrt{c} \tag{19}$$

$$p = \sqrt{a - b^2/4c}. \tag{20}$$

Note that a minimum sequence of three observations (three pings) is needed to solve the system.

The presence of square roots reminds us of the unfortunate possibility that through imprecise observations the fitted trajectory plotted in (u,v) coordinates might include negative values of v in violation of dual-beam theory. In particular, p and t should be real numbers, although p is not actually needed in continuing to compute F_1. Thus, we should check the reality conditions $c > 0$ and $4ac > b^2$ and continue only if these are true. The problem does not reside in an inability of the model to handle imprecise data, but rather in the use of a linear regression to fit the v_i's. If we were to regard p, t, and Δt directly as the unknowns in a least squares analysis instead of transforming to the unknowns a, b, and c, we could then approximately fit the model to imprecise data and be in no danger of violating dual-beam theory. The price of this increase in robustness would be the much more computationally intensive nonlinear regression analysis. We adhere to the linear method in order to keep the data analysis on a more familiar level in this introductory paper.

With t_i now given, a linear regression of u_i on t_i and t_i^2 according to eq. (15) will give best values of A, B, and C. The latter plugged into eq. (6) finally produce the maximum dorsal aspect TS F_1.

Scale analysis of the final steps in the computation of F_1 leads to another condition of acceptability of target data. Let m be some measure of the dispersion of t in the field of view, so that m is of the order of the size of the field of view expressed in radians. If $\langle u \rangle$ and Δu are, respectively, measures of the average and dispersion of u in the field of view, then the second-order linear regression yields the following scales:

$$A \sim \langle u \rangle$$
$$B \sim \Delta u/m$$
$$C \sim \Delta u/m^2.$$

When we use these in eq. (6), we find that F_1 scales in two distinctly different ways, depending on the sign of C:

If $C < 0$, then $(F_1 - A) \sim \Delta u$
If $C > 0$, then $(F_1 - A) \sim \Delta u/m^2$.

It can be shown that the value t_0, for which these maximum TS's are obtained, correspondingly scales in two distinctly different ways:

If $C < 0$, then $t_0 \sim m$

If $C > 0$, then $t_0 \sim 1/m$.

That is, when C is negative, the value of F_1 differs from the average observed TS by the order of the dispersion of the observed values, and this value occurs at an effective extrapolation distance within or beyond the field of view that is of the order of the size of the field of view. These are reasonable conditions for a credible extrapolation. However, when C is positive, the scale values of F_1 and t_0 are increased by a factor $1/m^2$, which is of the order of 100. Extrapolation of this order leads to shaky values so that the solution is only acceptable when C is negative.

The explanation for this mathematical property lies in the use of a linear regression to fit the data. Using a nonlinear least squares method, and fixing the value of $F_1 - F_0$, would yield a more moderately scaled value of C. In analyzing observed trajectories, we have indeed found that the results split into two distinct classes according to the sign of C, with some clearly unreasonable and some clearly reasonable values, and very few in-between cases. We have therefore rejected data giving positive C and continued with the computationally simpler fitting involving the linear regressions.

Parabolic fits have been made to four examples of field data using the methods described above. The data points and fitted parabolas are shown in Fig. 6. Values of F_1 were computed from eq. (6). In these examples, the point of maximum TS for the haddock (*Melanogrammus aeglefinus*) in Fig. 6D occurs near the acoustic axis, while in the other examples the dorsal maximum occurs in the field of view but near the margin of the sonified cone.

The Case of Three Pings

In the case $n = 3$ where a single fish is identified in just three pings, the linear regressions involving first v_i and then u_i reduce to algebraic fits through the points. Here, we outline the method of fitting.

Starting with the definition

$$(21)\quad d = (v_1 - 2v_2 + v_3)/s$$

we can write the reality condition as

$$d > 0$$

$$4v_1v_3 > (v_1 - 4v_2 + v_3)^2.$$

If these are true, then

$$(22)\quad \Delta t = \sqrt{d/2}$$

$$(23)\quad t_2 = (v_3 - v_1)/4s\,\Delta t$$

$$(24)\quad C = (u_1 - 2u_2 + u_3)/d$$

$$(25)\quad B = [(u_3 - u_1)\,\Delta t/d] - 2t_2C$$

$$(26)\quad A = u_2 - t_2\,(B + t_2C)$$

and finally, eq. (6) repeated here for convenience:

$$(6)\quad F_1 = (2A + C + \sqrt{B^2 + C^2})/2$$

which is the desired best estimate of the maximum dorsal TS of the fish target observed.

Discussion

With respect to the mathematical development, it is clear that if an ellipse is approximated by a parabolic fit, some information about the ellipse is lost. For example, a parabolic fit to the region of highest curvature is independent of the length of the major axis which is therefore irrecoverable from the elements of the parabola. In our case, what is lost is the possibility of recovering all five parameters (A, B, C, p, and s) occurring in the ellipse eq. (12). However, we have shown how and under what conditions the four parameters A, B, C, and p can be determined from the parabolic description of the trajectory. If then s is indeed a known system parameter, the ellipse may be recoverable in its entirety. We conjecture that essentially the same argument applies to the real system trajectory, or any conjectured other form. Knowledge of system beam pattern parameters such as s coupled with the measurable elements of the parabolic fit would effectively determine the trajectory.

Through the restricted field of view afforded by our acoustic system, only a small portion of the trajectory is available for analysis. Fitting a parabola to this small portion, which nearly always includes the neighbourhood of the vertex but sometimes not much more, is relatively easy. As can be seen from the data presented in Fig. 6, an acceptable parabolic curve is easily fit. By contrast, fitting an ellipse to the same data requires a sensitivity to very subtle variations in curvature unless nearly half or more of the ellipse is available. Thus, it is advantageous to accept s as a known system constant and use the parabolic fit.

Within the context of the two-parameter swimbladder model the real trajectory may be a parabola provided two improbable conditions are met. The beam pattern in decible form must be $s \tan^2 \theta$ and the swimbladder longitudinal axis must be vertical (parallel to the transducer axis). As a result, we recognize that it is virtually always true that the parabolic fit is at best an approximation to the real thing.

The information available in an EL plot is the same in both the real and idealized beam pattern systems, since they are indistinguishable for small angles. If we are confident that a reasonably good parabolic fit can be made from the available small angle information, then we are equally confident that we can construct the ellipse that would be generated from the full range of angles in the idealized system. The trajectory in its entirety in the real system might depart radically from an ellipse at large angles, but this complication is irrelevant to the argument. Searching the elliptical trajectory for the point of maximum target strength gives the idealized system version of F_1. But since F_1 is a property of the target and not of the system, it is the same for both systems and can therefore be identified as the maximum dorsal aspect TS.

A given elliptical trajectory in the idealized system also has a point with a minimum TS corresponding to the negative square root in eq. (6). This can be interpreted only as an upper bound on F_0. The projected apparent line of motion of the transducer relative to the fish is statistically unlikely to intersect the modeled swimbladder longitudinal axis where F_0 would be observed.

Should the parabolic fit in (u,v) coordinates have negative values of v which would happen if we accepted $p^2 < 0$, a violation of dual-beam theory is evident. The cause may be acoustic noise, a poor method of fitting imprecise data, observations of more than one target, a departure from straight-line motion, or an appreciable departure from the two-parameter axially symmetric model. Whatever the cause, the data cannot be ana-

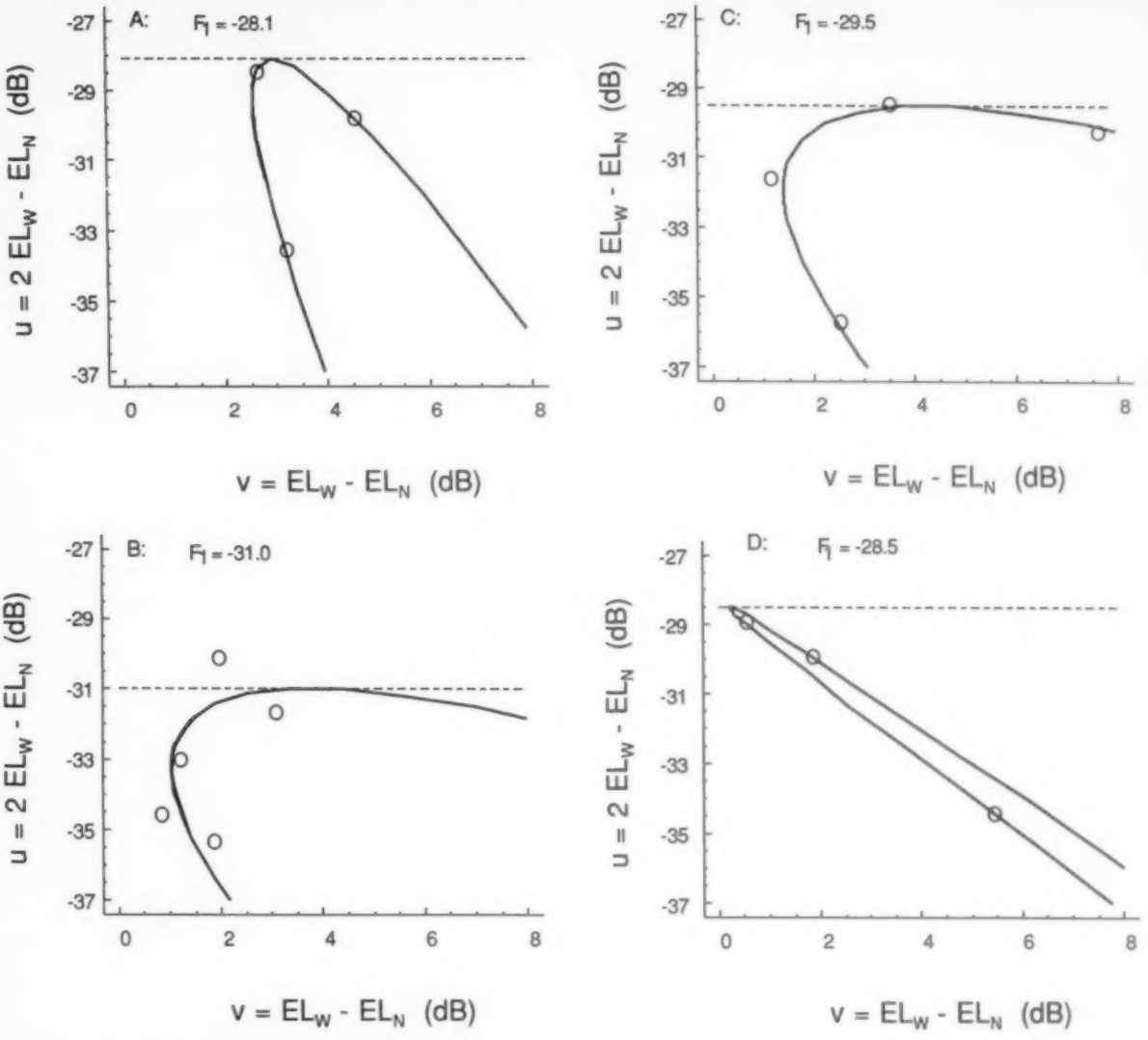


FIG. 6. Four trajectories from field data collected with ECOLOG I. (A and B) Pollock observed on the eastern Scotian Shelf; (C and D) haddock observed on the western Scotian Shelf. Broken lines indicate (v, F_1).

lyzed further and the maximum TS cannot be found with this method. Conversely, in practical terms it should be noted that failure of a given set of points to be fitted by a parabola with all positive values may be a powerful check on the adequacy of the algorithm used to identify a given set of data points as belonging to an acceptable single fish target.

Finally, we note in the plots of both actual field data and the modeled trajectories that EL trajectories and corresponding TS functions are highly variable. It may be possible to identify the sources of variability which are related to both the characteristics of the fish and to its behaviour. For example, properties of the trajectories, such as are seen in Fig. 6, clearly reflect information on fish aspect angle and orientation. With sufficiently precise data and fitting algorithms it may be possible to estimate the particular function of TS with aspect angle from the trajectory data. While the present model does not explicitly resolve information on the swimbladder aspect parameters θ' and φ', it may be possible to do so with further development. The possibility of extracting and using such information to characterize important biological attributes of fish is a subject for continued research.

Previous approaches to estimating a standard TS from field data have involved finding some "best" central measure such as an average of the beam pattern corrected observations (Traynor and Ehrenberg 1979; Foote 1987). Consideration of the form of the idealized trajectories together with a detailed understanding of their physical and mathematical basis suggests that some form of extrapolation from the observations is more appropriate. Using detailed data and appropriate methods, we have shown how F_1 can be estimated and that it offers a realistic alternative. Full verification of the general practicality of these particular techniques rests on the analysis of additional field

data. Nevertheless, similar procedures would appear to hold much promise for providing relevant, and more accurate, biological information on natural fish stocks.

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Morphological Differences between Hatchery and Wild Populations of Coho Salmon (*Oncorhynchus kisutch*): Environmental versus Genetic Origin

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Previous studies have demonstrated morphological differences between hatchery-reared coho salmon (*Oncorhynchus kisutch*) of hatchery origin and wild-reared coho of wild origin. We tested for a genetic component to this divergence by comparing coho from hatchery and wild populations both reared in the same hatchery environment and for an environmental component by comparing hatchery- and wild-reared coho both of wild origin. As in the previous studies, wild-reared fish from wild populations had greater head dimensions, larger median fins, and deeper bodies than did hatchery-reared fish from hatchery populations. This difference, summarized by the first principal component (PC1) of the size-adjusted data, was related to rearing environment rather than to genetic differences between hatchery and wild populations. Genetic divergence (or maternal effects) did occur between hatchery and wild populations along PC2 and PC3, but this divergence was slight compared with the environmentally induced differences between the two types of fish along PC1.

Les résultats d'études antérieures ont révélé des différences morphologiques entre des saumons cohos (*Oncorhynchus kisutch*) d'élevage vivant en milieu artificiel et des saumons cohos sauvages vivant en milieu naturel. Les auteurs ont tenté d'identifier une composante génétique expliquant cette différence en comparant des cohos d'élevage et sauvages élevés dans les mêmes conditions artificielles, ainsi qu'une composante environnementale en comparant des cohos sauvages vivant en milieux artificiel et naturel. Comme dans le cas des études antérieures, les poissons sauvages du milieu natural montrent des valeurs plus élevées des caractéristiques morphométriques de la tête, des nageoires médianes plus grandes et une profondeur du corps plus élevée que les poissons d'élevage du milieu artificiel. Cette différence, résumée sous la forme d'une première composante principale (PC1) des données ajustées selon la taille, était liée au milieu de croissance plutôt qu'à des différences génétiques entre les populations d'élevage et sauvage. Les PC2 et PC3 ont révélé une divergence génétique (ou hérédité cytoplasmique) entre les populations d'élevage et sauvage, mais elle était faible par rapport aux différences entre les deux catégories de cohos révélées par la PC1 et entraînées par le milieu.

Received July 23, 1990
Accepted March 13, 1991
(JA659)

Reçu le 23 juillet 1990
Accepté le 13 mars 1991

Body morphology is believed to be under conflicting selectional pressures in fishes (e.g. Riddell and Leggett 1981; Taylor and McPhail 1985a; Fleming and Gross 1989; Swain and Holtby 1989). Body shape affects swimming performance, but the optimal shape differs between burst and sustained swimming modes (Webb 1978, 1984; Taylor and McPhail 1985b). A deep body and large median fins provide superior burst swimming performance whereas a fusiform or "streamlined" shape is superior for sustained swimming. Body shape may also affect performance during agonistic interactions, with a deep body and large median fins conferring an advantage during aggressive displays (cf. Swain and Holtby 1989). Thus, selection for sustained swimming performance is opposed by selection for burst swimming and perhaps agonistic performance.

Coho salmon (*Oncorhynchus kisutch*) typically spend their first year in fresh water, either in streams where they are territorial or in lakes where they are schooling. Juveniles then migrate to the ocean where they spend about 18 mo growing before they return to their natal streams to establish spawning territories, spawn, and die. During stream residence, both as territorial juveniles and as spawning adults, selection may be predominantly for burst swimming and agonistic performance. Burst swimming facilitates escape from predators and success in competition for drift food, in intrasexual competition, and in spawning (e.g. Fleming and Gross 1989). During migration and residence in open waters, selection may be predominantly for sustained swimming performance. Differences in morphology between geographic (coastal versus interior) and ecological (stream versus lake rearing) types of coho salmon are consistent with these predictions. Coho from interior or lake-rearing populations are more streamlined, with shallower bodies and smaller median fins, than are those from coastal or stream-rearing populations (Taylor and McPhail 1985a; Swain and Holtby 1989). The differences between interior and coastal populations are known to have a genetic basis (Taylor and McPhail 1985a).

Coho salmon are reared extensively in hatcheries to supplement natural production. A widespread concern is that selection in the novel hatchery environment may result in an evolutionary response away from the wild type (e.g. Reisenbichler and McIntyre 1977; Helle 1981; Larkin 1981; Ryman and Stahl 1981). Wild and hatchery populations of coho salmon do differ in body morphology, both as juveniles (Hjort and Schreck 1982; Taylor 1986) and as adults (Fleming and Gross 1989). At both stages, the observed differences are in the direction predicted for evolutionary responses to selection in the hatchery environment. For example, Fleming and Gross (1989) predicted the evolution of a more streamlined body shape in hatchery populations due to reduced selection for burst swimming performance (because juvenile rearing occurs in a controlled predator-free environment and spawning is artificial). Hatchery fish are indeed more streamlined than wild fish, and Fleming and Gross (1989) claimed that this difference indicates an evolutionary response by hatchery populations to novel selection pressures in the hatchery environment. However, body shape is known to be labile to environmental influences in fishes (e.g. Martin 1949; Winans 1984; Meyer 1987; Currens et al. 1989; Beacham 1990), and Taylor (1986) suggested that morphological divergence between hatchery and wild coho salmon may be largely environmentally induced.

In this paper we test for genetic and environmental components to morphometric differences between hatchery and wild juvenile coho salmon. Previous studies cannot distinguish between the two sources of variation because comparisons have been between hatchery-reared fish from hatchery populations and wild-reared fish from wild populations. We compare body shape between hatchery and wild juveniles reared from fertilization in the same hatchery environment to test for a genetic component to divergence and between hatchery- and wild-reared juveniles from the same wild populations to test for an environmental component to divergence.

Material and Methods

Material

In November and December 1988, eggs (about 100 per female) and milt were collected from six hatchery and six wild populations of coho salmon (Fig. 1) according to the schedule in Table 1. (Two adjacent wild populations, Stamp tributaries 1 and 2, were combined into one group for hatchery rearing due to limited rearing facilities and the small numbers of parent fish collected from each tributary.) All wild populations were of the typical stream-rearing type. Hatchery populations had been established for four to five generations in five cases and two generations in one case (Chilliwack Hatchery).

At the Pacific Biological Station, Nanaimo, all eggs collected from one population on one day were combined and washed in a sodium bicarbonate solution (13.68 g/L) to enhance fertilization (Wilcox et al. 1984). These were then divided into as many lots as there were males from that population, and each lot was fertilized by the milt of one male. After water hardening, paternal groups from the same population were combined and embryos from each population were reared in separate baskets in the same stack of Heath trays at 5°C. After 375°d of development, embryos were transferred to Rosewall Creek Hatchery for the completion of development at 8°C. A random sample of 100 alevins was taken from each population and ponded in a separate tank 0.75 m high and 0.9 m in diameter, filled to a volume of 350 L with a water flow of 9–13.5 L/min. The fish were reared on a diet of commercial dry crumble under a simulated natural photoperiod at a water temperature of 8°C. When the average standard length (SL) reached 50 mm, all individuals in a population were killed in 2-phenoxyethanol and frozen for later measurement.

In June 1989, wild-reared juveniles were collected by seining from five of the six wild populations and frozen for later measurement. (Collections were not made from Chef Creek due to a transplant of hatchery-reared fry in 1989.)

The three types of fish are referred to hereafter as follows: hatchery fish reared in a hatchery, HH; wild fish reared in a hatchery, WH; and wild fish reared in the wild, WW. Standard length was similar among the three fish types (mean ± SD): 49.2 ± 2.7 mm for HH ($N = 178$), 48.6 ± 3.4 mm for WH ($N = 150$), and 48.4 ± 4.7 mm for WW ($N = 143$).

Measurements

Morphometric measurements were made on about 30 fish from each of the 16 populations (six HH, five WH, and five WW; $N = 30$ in 12 cases, 29 in three cases, and 24 in one case). (The two Chef Creek groups were combined into one group in proportion to the number of crosses contributing to each of the original groups, with 10 randomly selected fish measured from the November 25 group and 20 fish measured from the November 29 group). Measurements included lengths of the leading edges of the dorsal and anal fins (fin heights), caudal peduncle length (after Hubbs and Lagler 1958), and the truss network, a system of vertical, horizontal, and oblique distances between anatomical landmarks (Fig. 2; Strauss and Bookstein 1982). Measurement procedures were the same as those described by Winans (1984) and Swain and Holtby (1989). Fish were thawed and placed on water-resistant paper. Landmark positions were recorded by making holes with a dissecting needle in the water-resistant paper alongside the location of each landmark. Coordinates of each landmark were recorded to within 0.1 mm using a high-resolution digitizer. Distance measurements were then calculated from the coordinates.

Morphometric characters are referred to here either generally using descriptive phrases such as "caudal peduncle length" or "body depth" or precisely using the numbered landmarks in Fig. 2. Note that the same general description may apply to more than one character. For example, distances 9–11, 10–12, and 9–15 are all described as caudal peduncle lengths.

Statistical Analysis

Analyses were performed using the Statistical Analysis System (SAS Institute Inc. 1982). All analyses were of $\log_{10}$-transformed measurements. These were standardized to a grand mean body length of 48.6 mm. When slopes of the regression of the log-transformed trait against the logarithm of SL were homogeneous among populations, this size adjustment used the common within-groups slope obtained from analysis of covariance of the following model:

$$D_{ijk} = \mu + A_i + P_{ij} + \beta X_{ijk} + e_{ijk}$$

where A_i is the effect of fish type i (HH, WH, or WW), P_{ij} is the effect of population j nested within fish type i, D_{ijk} and X_{ijk} are the $\log_{10}$-transformed morphometric measurement and SL of fish k in population j of type i, and e_{ijk} is a random normal deviation. When slopes were significantly ($P < 0.05$) heterogeneous among populations (truss elements 4–5, 8–10, 9–10,

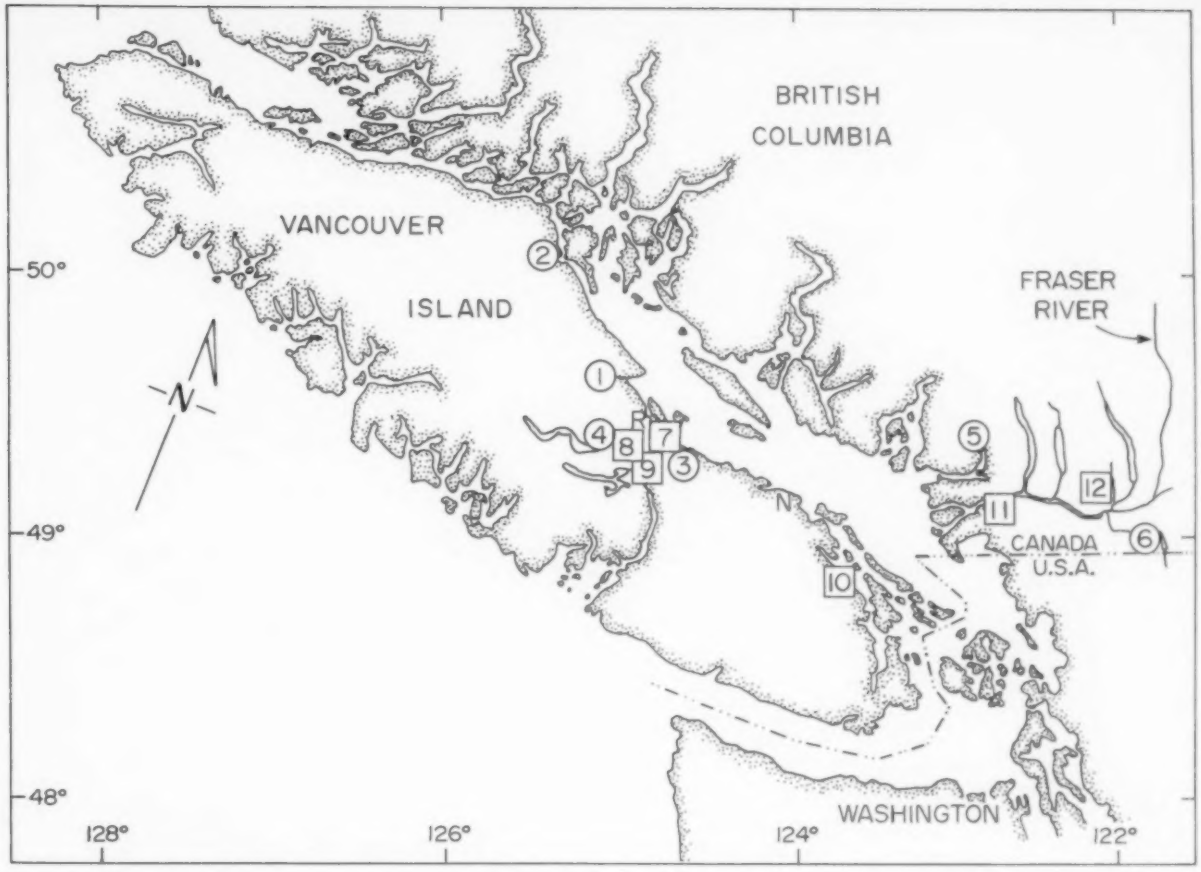

FIG. 1. Locations of hatchery (circles, 1–6), and wild (squares, 7–12) populations of coho salmon sampled in this study: 1 = Puntledge; 2 = Quinsam; 3 = Big Qualicum; 4 = Robertson Creek; 5 = Capilano; 6 = Chilliwack; 7 = Chef Creek; 8 = Stamp River tributary 1; 9 = Stamp River tributary 2 (Beaver Creek); 10 = Bonsall Creek; 11 = West Creek; 12 = Weaver Creek. N = Nanaimo, British Columbia; R = Rosewall Creek Hatchery.

TABLE 1. Sources of hatchery-reared juvenile coho salmon, including numbers of fish from which gametes were taken.

Population	Type	Date	♀	♂
Puntledge	Hatchery	Nov. 28	10	10
Quinsam	Hatchery	Nov. 7	10	10
Big Qualicum	Hatchery	Dec. 6	10	10
Robertson Creek	Hatchery	Nov. 16	8	10
Capilano	Hatchery	Nov. 8	8	10
Chilliwack	Hatchery	Nov. 17	10	10
Chef Creek[a]	Wild	Nov. 25	1	2
		Nov. 29	2	3
Stamp tributary 1[b]	Wild	Nov. 29	1	5
Stamp tributary 2[b]	Wild	Nov. 29	4	5
Bonsall Creek	Wild	Dec. 13	6	5
West Creek	Wild	Nov. 30	5	7
Weaver Creek	Wild	Dec. 7	6	5

[a]Gametes collected on different dates reared separately.
[b]Combined into one group at fertilization.

9–11, 10–11, 9–12, 11–12, 12–13, and 13–14, caudal peduncle length 9–15, and dorsal fin height 6–17), adjustment used the separate within-population slopes.

Univariate morphometric differences among fish types were tested by ANOVA of the adjusted data with fish type and population nested within fish type as effects. Tests of the effect of fish type used the Type III mean square for the effect of population nested within fish type as the error term (df = 2,13). Pairwise *t* tests between fish types used this same error mean square. Because there are only three means in these pairwise comparisons, the experimentwise error rate was held to the 0.05 level by making pairwise comparisons only if the overall ANOVA *F* test was significant at this level (Goodnight et al. 1982).

Morphometric variation was summarized by extracting principal components (PC) from the correlation matrix of the adjusted data. Because these components were extracted from size-adjusted data, PC's, including the first component, described shape variation in this analysis. This is confirmed by the lack of correlation between each PC and SL within groups ($r^2 < 0.002$ for PC1; $r^2 < 0.003$ for PC2; $r^2 < 0.006$ for PC3). Differences in PC scores were tested among fish types as described above for univariate differences. Confidence ellipses were calculated following Owen and Chmielewski (1985).

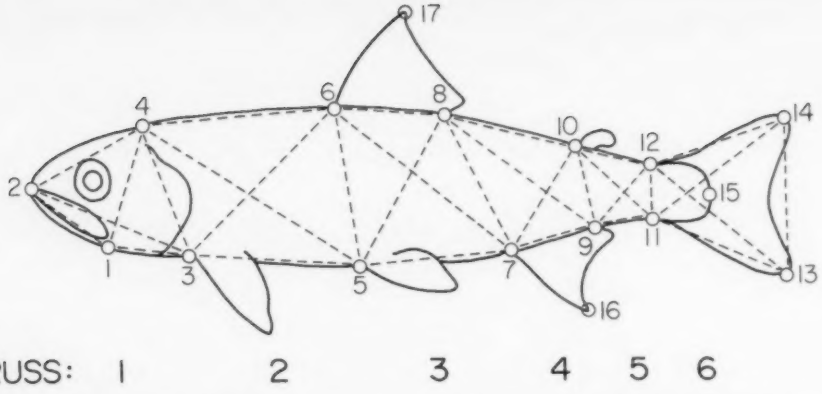

FIG. 2. Locations of the 17 landmarks used to calculate the truss network (broken lines), caudal peduncle length (9–15), anad fin height measurements. The landmarks are described in detail in Winans (1984), with modifications given in Swain and Holtby (1989). Trusses are numbered 1–6.

The optimal procedure for size adjustment in morphometric analyses is currently a matter of debate (e.g. Rohlf and Bookstein 1987; Rohlf 1990). To confirm that our conclusions were not contingent on the procedure used for size adjustment, we also performed an analysis using sheared principal components of the correlation matrix of the unadjusted data (Bookstein et al. 1985). This analysis led to the same conclusions as the PC analysis of the adjusted data. We present results of the latter analysis here, rather than those of the analysis using shearing, because an assumption of homogeneity of slopes is required for shearing but is violated for some of the traits examined here.

Results

Univariate differences among the three fish types were significant for 22 of the 34 morphometric measurements (Table 2). Differences were mostly related to rearing environment. For 15 of the 22 characters, hatchery and wild fish did not differ significantly when reared in a hatchery environment whereas both hatchery-reared types (HH and WH) differed significantly from wild-reared fish (WW). Wild-reared fish tended to have relatively longer and deeper heads, relatively shorter midbody lengths, relatively deeper bodies, and relatively larger median fins than did hatchery-reared fish (Table 2).

Although morphological differences among fish types were predominantly environmental, univariate comparisons did suggest some genetic divergence between hatchery and wild types. For three characters (6–7, 7–8, and 9–10), differences were significant only between HH and WW fish, suggesting that both genetic and environmental differences contributed to the relatively slight variation among fish types for these characters. Both genetic and environmental differences also contributed to variation in height and upper lobe length (12–14) of the caudal fin. For caudal fin height, genetic and environmental effects were both strong and in the same direction, so that WH fish were intermediate between HH and WW fish and differences between all fish types were significant. For upper lobe length of the caudal fin, genetic and environmental effects were strong and in opposing directions, so that no significant differences occurred between HH and WW fish but WH fish differed significantly from both other types. Finally, differences were entirely genetic for two characters associated with caudal peduncle length (9–11 and the oblique element 10–11), with wild fish having greater lengths. In summary, of the 22 significant univariate differences, 15 were entirely environmental, five had both environmental and genetic components, and only two were entirely genetic.

Morphometric differences among the three fish types are summarized by the first three PC's of the correlation matrix (Table 3). The greatest differences among fish types were on PC1 (Table 4; Fig. 3). This component summarized differences related to rearing environment. Mean PC1 score did not differ significantly between the HH and WH groups but did differ significantly between both these groups and the WW group (Table 4). The characters correlated most strongly with this component were those related to head size, median fin size, and body depth. Individuals with relatively large heads, relatively great median fin heights, and relatively deep bodies had high scores on PC1. Mean PC1 score was highest for the WW group.

PC2 and PC3 summarized differences between fish types that were both genetic and environmental in origin. PC2 was mainly a contrast between caudal peduncle size (variables with large positive loadings on PC2) and median fin size (variables with large negative loadings on PC2). Genetic and environmental effects were in opposing directions along PC2, so that no significant differences occurred between HH and WW fish whereas WH fish differed significantly from both other groups (Table 4). WH fish had relatively high PC2 scores, indicating proportionally large caudal peduncles in relation to median fin size in this fish type. PC3 contrasted anterior body length (in truss 2) and caudal fin lobe length on the one hand (variables with large positive loadings on PC3) with head length, caudal peduncle length 9–11, and caudal fin height on the other hand (variables with large negative loadings on PC3). This contrast was primarily between caudal fin lobe length 11–13 and caudal peduncle length 9–11. Both genetic and environmental effects were weak and in the same direction along PC3, so that WH fish were intermediate in PC3 score and differences were significant only between the extreme HH and WW groups. Although these differences among fish types along PC2 and PC3 were significant (Table 4), they were slight compared with the separation of WW fish from the other fish types along PC1 (Fig. 3).

TABLE 2. Mean body measurements of juvenile coho salmon from hatchery and wild populations. Measurements adjusted to 48.6 mm SL. Morphometric characters are the distances between the landmarks numbered in Fig. 2. Fish types: HH, hatchery-reared hatchery fish; WH, hatchery-reared wild fish; WW, wild-reared wild fish. Only distances that differed significantly between fish types are shown. P is from the F test comparing mean squares due to fish type and to populations nested within fish types (df = 2,13). Fish types coded with different letters differ significantly ($P < 0.05$) according to the t test described in the text.

Morphometric character	Measurement (mm)				Pairwise t test			
	HH	WH	WW	P	HH	WH	WW	Description
1–2	7.69	7.89	8.42	0.0008	A	A	B	Premaxilla length
2–3	12.72	12.89	13.37	0.0008	A	A	B	Snout to pectoral fin
1–4	9.73	9.91	10.69	0.0001	A	A	B	Head depth 1
2–4	9.83	9.87	10.50	0.0001	A	A	B	Forehead length
3–4	11.02	11.21	11.65	0.0004	A	A	B	Head depth 2
4–6	15.21	15.27	14.43	0.0001	A	A	B	Head to dorsal fin
5–6	12.82	12.96	13.61	0.0001	A	A	B	Body depth 1
5–7	8.60	8.80	8.18	0.0002	A	A	B	Pelvic to anal fin
6–7	15.60	15.78	15.88	0.03	A	AB	B	Oblique element, truss 3
5–8	12.29	12.29	12.79	0.0001	A	A	B	Oblique element, truss 3
7–8	11.28	11.41	11.60	0.02	A	AB	B	Body depth 2
7–9	7.00	6.87	7.36	0.001	A	A	B	Anal fin base
7–10	10.52	10.51	11.09	0.0001	A	A	B	Oblique element, truss 4
9–10	6.87	6.99	7.07	0.04	A	AB	B	Caudal peduncle depth 1
9–11	2.97	3.21	3.28	0.02	A	B	B	Caudal peduncle length
10–11	7.90	8.21	8.25	0.009	A	B	B	Oblique element, truss 5
12–13	16.01	15.86	16.38	0.007	A	A	B	Caudal fin length, lower lobe
11–14	15.65	15.45	16.01	0.002	A	A	B	Caudal fin length, upper lobe
12–14	13.15	12.81	13.27	0.03	A	B	A	Caudal fin length, upper lobe
13–14	13.33	13.98	15.54	0.0001	A	B	C	Caudal fin height
6–17	9.81	9.41	11.75	0.0001	A	A	B	Dorsal fin height
7–16	8.52	8.54	11.86	0.0001	A	A	B	Anal fin height

PC4 and PC5 accounted for 7 and 6%, respectively, of the total morphometric variation. Mean scores along these components did not differ among fish types ($P > 0.3$).

Discussion

The same morphometric differences between wild-reared coho salmon of wild origin (i.e. type WW) and hatchery-reared coho salmon of hatchery origin (i.e. type HH) were observed in this study and in previous studies on juveniles (Taylor 1986) and adults (Fleming and Gross 1989). In all three cases, the wild salmon had larger head dimensions, larger median fins, and greater body depths than did hatchery salmon. A more streamlined body shape (i.e. shallower body and smaller median fins) is expected to be favoured in hatchery fish due to reduced selection for burst swimming performance (Fleming and Gross 1989). Thus, the observed differences in morphology between hatchery and wild coho are in the direction predicted for evolutionary responses to selection. However, we found that these differences, summarized here by variation in the first principal component of the size-adjusted data, clearly reflected an effect of the rearing environment rather than a genetic difference between hatchery and wild populations. Thus, there is no evidence of substantial genetic divergence in juvenile morphology in response to the predicted differences in phenotypic selection between hatchery and wild populations of coho salmon. Furthermore, the claim by Fleming and Gross (1989) for an evolutionary response in *adult* morphology to novel selection pressures in hatchery populations must also be viewed with caution. Differences in adult morphology are the same as the environmental differences observed in juveniles. Taylor and McPhail (1985a) have demonstrated that population-specific traits in juveniles may be maintained in adults. Thus, the differences in morphology between hatchery and wild adults may reflect environmental differences established in juveniles. Environmental differences established in juveniles may also be reinforced by environmental differences between adults in the marine environment. While similar ranges of environments are available to both hatchery and wild adults, environmental differences (e.g. in feeding ecology) may persist between the two types in the marine phase of the life cycle if permanent behavioural differences exist between the two types (e.g. Swain and Riddell 1990).

The differences between hatchery and wild juveniles that we describe as environmental may derive from two sources. First, these differences may reflect phenotypic plasticity, a modification of development in response to differences in rearing environment. Second, they may result from selective mortality of juveniles in the wild. (Mortality was negligible among hatchery-reared juveniles in this study.) If selective mortality among wild-reared juveniles is the major cause of these differences, then it should be possible to generate the phenotype distribution of wild-reared juveniles by selection from the distribution among hatchery-reared juveniles. That is, phenotypes of juveniles surviving in the wild should be a subset of those observed among hatchery-reared juveniles. This is clearly not the case in our data: a high proportion of wild-reared juveniles had phenotypes more extreme than any observed among hatchery-reared juveniles (Fig. 4). Thus, selective mortality is not supported as an important cause of the environmental differences in morphology reported here. On the contrary, morphological plasticity has been demonstrated previously in salmonids (e.g Martin 1949; Currens et al. 1989; Beacham 1990), and phenotypic plasticity appears to be the main cause of the morphological differences between hatchery- and wild-reared juveniles in this study.

TABLE 3. Loadings of the morphometric characters on the first three PC's. Characters are the distances between the landmarks numbered in Fig. 2.

Character	PC1	PC2	PC3	Description
1–2	0.230	0.072	−0.151	Premaxilla length
1–3	0.007	−0.089	−0.098	Premaxilla to pectoral fin
2–3	0.202	−0.002	−0.184	Snouth to pectoral fin
1–4	0.286	−0.005	−0.134	Head depth 1
2–4	0.204	−0.142	−0.175	Forehead length
3–4	0.269	0.069	−0.018	Head depth 2
3–5	−0.020	−0.075	0.113	Pectoral to pelvic fin
4–5	0.057	0.050	0.245	Oblique element, truss 2
3–6	0.151	0.134	0.219	Oblique element, truss 2
4–6	−0.136	0.160	0.276	Head to dorsal fin
5–6	0.294	0.060	0.124	Body depth 1
5–7	−0.121	0.162	0.092	Pelvic to anal fin
6–7	0.182	0.167	0.238	Oblique element, truss 3
5–8	0.260	0.016	0.117	Oblique element, truss 3
6–8	0.018	−0.192	−0.029	Dorsal fin base
7–8	0.218	0.197	0.249	Body depth 2
7–9	0.094	−0.239	−0.139	Anal fin base
8–9	0.101	0.210	0.193	Oblique element, truss 4
7–10	0.246	−0.088	−0.046	Oblique element, truss 4
8–10	0.017	0.085	0.102	Dorsal to adipose fin
9–10	0.190	0.244	0.065	Caudal peduncle depth 1
9–11	0.056	0.194	−0.313	Caudal peduncle length 1
10–11	0.148	0.295	−0.149	Oblique element, truss 5
9–12	0.117	0.267	−0.068	Oblique element, truss 5
10–12	−0.014	0.282	−0.107	Caudal peduncle length 2
11–12	0.182	0.138	0.066	Caudal peduncle depth 2
11–13	0.036	−0.198	0.359	Caudal fin length, lower lobe
12–13	0.190	−0.231	0.159	Caudal fin length, lower lobe
11–14	0.176	−0.195	0.235	Caudal fin length, upper lobe
12–14	0.098	−0.298	0.182	Caudal fin length, upper lobe
13–14	0.194	−0.020	−0.178	Caudal fin height
9–15	0.030	0.074	−0.139	Caudal peduncle length 3
6–17	0.221	−0.216	−0.050	Dorsal fin height
7–16	0.251	−0.185	−0.145	Anal fin height
% of total variance	25.0	11.5	9.3	

TABLE 4. Mean PC scores of the three types of juvenile coho salmon. *P* is from the *F* test comparing variation among fish types with variation among populations nested within fish types (df = 2,13). Fish types coded with different letters differ significantly ($P < 0.05$) according to the *t* test described in the text.

Fish type	PC1	PC2	PC3
HH	−1.78 A	−0.46 A	0.70 A
WH	−1.09 A	1.24 B	0.04 AB
WW	3.37 B	−0.73 A	−0.92 B
P	0.0001	0.05	0.02

Genetic divergence in physiological, behavioural, and other performance traits has been demonstrated between hatchery and wild populations of salmonids, including coho salmon (e.g. Greene 1952; Vincent 1960; Flick and Webster 1964; Moyle 1969; Reisenbichler and McIntyre 1977; Bachman 1984; Swain and Riddell 1990). Morphological characters are also expected to be subject to different selection pressures between hatchery and wild environments (e.g. Fleming and Gross 1989), and evolutionary responses to local selection pressures have been demonstrated for morphological characters of coho salmon in other circumstances (e.g. coastal versus interior populations (Taylor and McPhail 1985a)). Why then has there been little evolutionary response by morphological characters of coho salmon to the expected differences in phenotypic selection between hatchery and wild populations? We studied hatchery populations that had been established for four to five generations in five cases and two generations in one case. One possibility is that there has not been sufficient time since the founding of these hatchery populations for detectable genetic changes to accrue. However, genetic differences appear to have developed between hatchery and wild populations in an equally brief time in other cases (Reisenbichler and McIntyre 1977; Swain and Riddell 1990). Another possibility is that, because of phenotypic plasticity, the intensity of selection is low in this case. We observed a large amount of environmental variation in the morphological characters that are presumed to be subject to differences in phenotypic selection between hatcheries and the wild (i.e. in the morphological variation summarized by PC1). We believe that this environmental variation reflects phenotypic plasticity rather than selective mortality in the wild. If predictions about the differences in phenotypic selection between hatchery and wild environments are correct, then this phenotypic plasticity is adaptive. As a result of environmentally induced variation in development, the same genotype produces a phenotype which appears to be adapted to the hatchery envi-

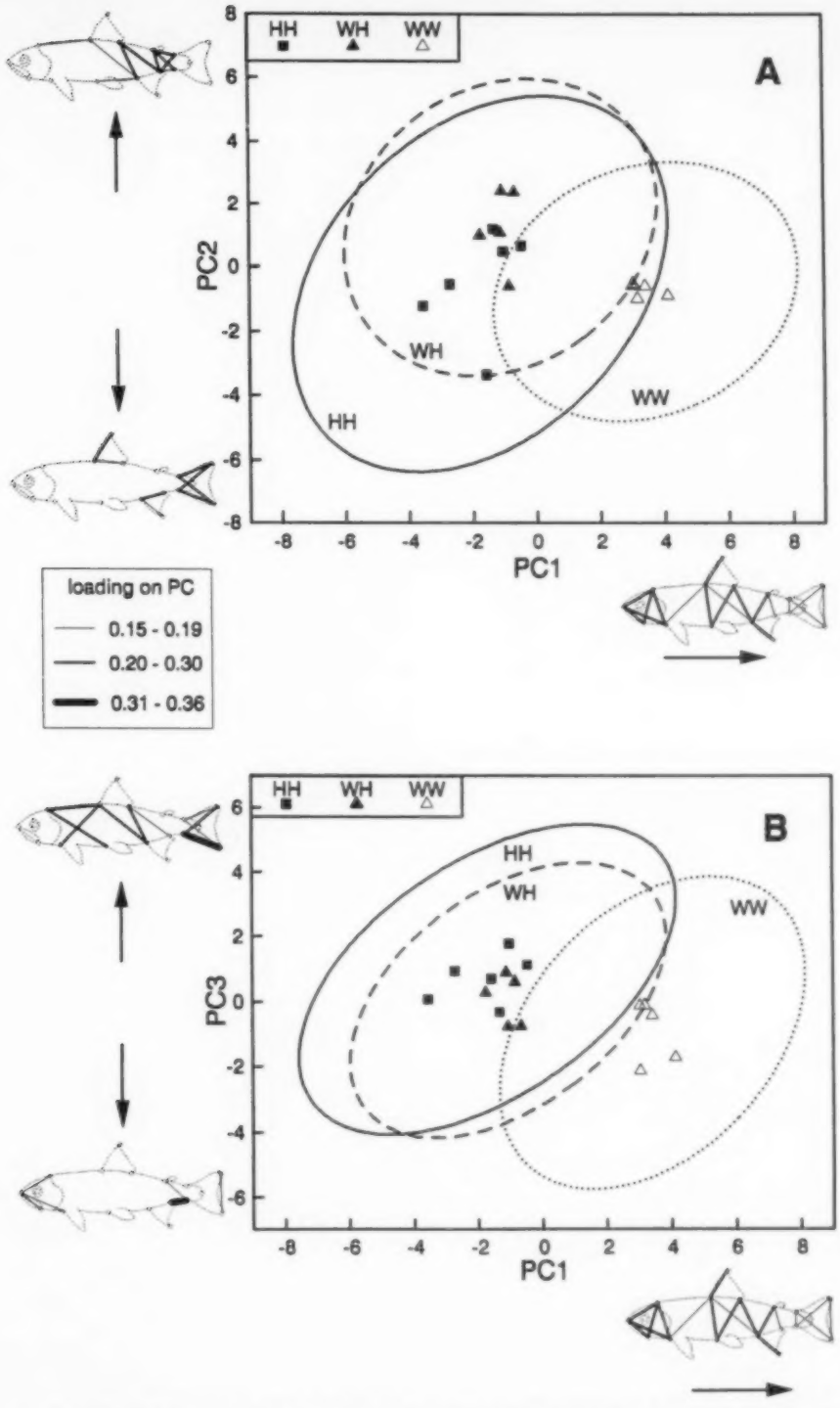

FIG. 3. Population centroids from a PC analysis of size-adjusted morphometric characters of juvenile coho salmon. Squares are centroids for hatchery populations and triangles for wild populations. Solid symbols indicate hatchery rearing and open symbols wild rearing. The 95% confidence ellipses are also shown for each of the three fish types. Characters loading strongly on each PC are depicted beside each axis. Arrows indicate the sign of the correlation between these characters and the respective PC. (A) PC2 against PC1; (B) PC3 against PC1.

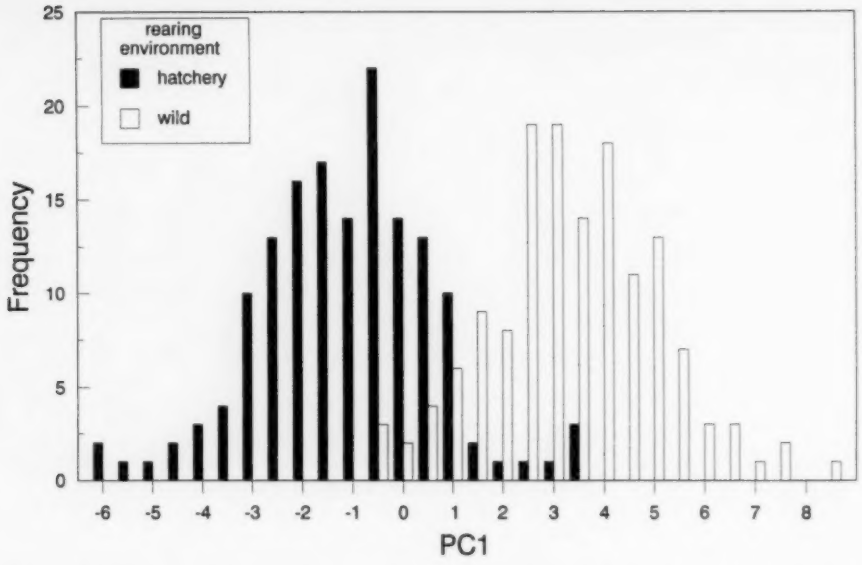

FIG. 4. Distribution of PC1 scores among wild coho salmon reared in the wild (open bars) or in a hatchery (solid bars).

ronment if development occurs in that environment, or a contrasting phenotype which appears to be adapted to the wild if development occurs in the wild. Thus, because of this plasticity, the wild genotype may be "preadapted" to the hatchery environment and may experience little directional selection away from the wild type in that novel environment. If so, this adaptive phenotypic plasticity may be fortuitous, or it may have evolved in the wild in response to natural variation in selection pressure parallel to that between typical wild and hatchery environments (e.g. between the typical stream and atypical lake rearing environments). A possible environmental cue for a switch in morphological development is growth rate (Martin 1949). Growth rate is high both in hatcheries (e.g. Fleming and Gross 1989) and in lake-rearing populations (L. B. Holtby, pers. comm.), and similar differences in juvenile morphology (i.e. in body depth and median fin size) occur between the slow-growing stream-rearing populations and the fast-growing hatchery or lake-rearing populations (Taylor 1986; Swain and Holtby 1989; this study). However, we emphasize that the adaptive significance suggested here for this phenotypic plasticity is speculative and can only be confirmed by direct empirical tests of the predicted differences in selection between hatchery and wild environments.

Differences between WH and HH fish have been described here as genetic differences because these two fish types experienced the same rearing environment. However, it is also possible that these differences are due to prefertilization environmental effects (e.g. environmental maternal effects) rather than genetic differences. Maternal effects on early survival, size, and growth rate have been demonstrated frequently in fishes and are generally attributed to effects of egg size (e.g. Fowler 1972; Ayles 1974; Gall 1974; Kanis et al. 1976). The prefertilization environment has also been shown to affect morphological characters in fishes (Dentry and Lindsey 1978; Swain and Lindsey 1986), although these characters were meristic traits which are affected by processes occurring soon after fertilization and are fixed early in life (typically before hatching or emergence). We do not expect a large effect of egg size or early embryonic processes on body shape of juveniles months after emergence. Thus, we believe that differences reported here between WH and HH fish are probably genetic differences, but more extensive experiments spanning two generations would be required to rule out the possibility of prefertilization environmental effects.

If differences between WH and HH fish are genetic, then this study does provide some evidence of genetic divergence between hatchery and wild coho salmon. This genetic divergence occurred along PC2 and PC3, and was slight compared with environmentally induced differences between hatchery and wild coho along PC1. Possible genetic divergence along PC3 achieved statistical significance only when reinforced by environmental differences in the same direction (i.e. only between WW and HH fish). Divergence along PC3 mainly reflected a tendency for hatchery fish to have large caudal fin lobe lengths 11–13 in relation to caudal peduncle length 9–11. The adaptive significance of this divergence between hatchery and wild fish is not known. It bears no clear relationship to the predicted differences in selection between the two fish types. Divergence along PC2 was stronger, achieving significance between WH and HH fish. However, this divergence did not appear to reflect an evolutionary response to the predicted differences in phenotypic selection between hatchery and wild fish. For example, smaller median fins are predicted to be favoured in hatchery populations (Fleming and Gross 1989), yet divergence along PC2 partly reflected a tendency for HH fish to have larger median fins than WH fish. Environmentally induced variation counteracted this possible genetic divergence along PC2, so that WW and HH fish did not differ in PC2 score. That is, the same phenotype (PC2 score) was produced by a hatchery genotype in a hatchery environment and by a wild genotype in a wild environment. Such genetic divergence without phenotypic divergence has been demonstrated previously between seasonal

ecotypes of chum salmon, *Oncorhynchus keta* (Tallman 1986, 1988). In this previous demonstration, genetic divergence appeared to be an adaptation to produce the same optimal phenotype in different developmental environments.

When phenotypic differences among populations are in the directions predicted by evolutionary theory, they are sometimes taken as evidence for genetic divergence in response to natural selection (e.g. Fleming and Gross 1989). This study demonstrates the danger in this practice. Phenotypic differences between hatchery and wild populations reared in their respective environments (summarized by PC1) were in the direction predicted by evolutionary theory but were mostly environmentally induced. However, probable genetic differences did occur between the two types of populations, but these were slight (PC3) or were not reflected by phenotypic differences between the two types when development occurred in their respective environments (PC2).

Acknowledgements

We thank S. Baillie, K. Simpson, and R. Kaufman for assistance in field work, hatchery staff for assistance in gamete collection, and fish culture staff at Rosewall Creek Hatchery for the incubation and rearing of juveniles. Dr. L. B. Holtby provided the digitizer and digitizing software. We thank L. B. Holtby, R. Claytor, and M. Chadwick for helpful discussion. Experiments and laboratory work were conducted while the senior author held a visiting fellowship with the Department of Fisheries and Oceans at the Pacific Biological Station, Nanaimo.

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Effect of Overwinter Mortality on Reproductive Life History Characteristics of Pumpkinseed (*Lepomis gibbosus*) Populations

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Fox, M. G., and A. Keast. 1991. Effect of overwinter mortality on reproductive life history characteristics of pumpkinseed (*Lepomis gibbosus*) populations. Can. J. Fish. Aquat. Sci. 48: 1792–1799.

Pumpkinseed (*Lepomis gibbosus*) inhabiting two shallow ponds that experienced major winterkills matured 1–2 yr earlier and at a smaller size (difference >20 mm in length) than conspecifics living in an adjacent, nonwinterkill lake. Pond females were more fecund relative to body weight, and their gonadosomatic index was about double that of lake females. Pond males showed a significant, but less drastic increase in gonadosomatic index over lake males. Winterkills occurred in different years in the two ponds, but in both cases, pumpkinseeds showed significantly lower age and size at maturity following winterkill in comparison with a preceding or subsequent nonwinterkill year. These results are consistent with life history predictions for organisms living in fluctuating environments and indicate that reproductive life history traits are very plastic in pumpkinseeds living in high-risk environments. Advanced maturity following winterkill may be the result of abundant food supply to survivors or release from social factors that prevent maturation of small pumpkinseeds.

Les crapets-soleil (*Lepomis gibbosus*) vivant dans deux étangs peu profonds qui ont subi d'importantes martégades deviennent matures de 1 à 2 ans plus tôt et à une taille inférieure (plus de 20 mm de différence de longueur) que des crapets-soleil vivant dans un lac adjacent non touché par la martégade. Les femelles des étangs présentaient une fécondité relative au poids corporel supérieure à celle des femelles du lac, et un indice gonadosomatique à peu près deux fois plus élevé. L'indice gonadosomatique des mâles des étangs était supérieur à celui des mâles du lac, de manière significative mais cependant moins marquée. Les deux étangs n'ont pas été touchés par la martégade la même année, mais dans les deux cas, l'âge et la taille des crapets-soleil à la maturité après la martégade étaient inférieurs de façon significative à l'âge et à la taille des poissons matures de l'année précédente ou suivante au cours de laquelle il n'y avait pas eu de martégade. Ces résultats confirment les prédictions concernant le développement des organismes vivant dans des milieux changeants, et indiquent que les modalités de la reproduction sont très souples chez les crapets-soleil vivant dans des milieux à risque élevé. La précocité de la maturation après une martégade peut être causée par l'abondance de la nourriture pour les survivants, ou par la disparition de facteurs sociaux qui retardent la maturation des jeunes crapets-soleil.

Received August 8, 1990
Accepted March 13, 1991
(JA673)

Reçu le 8 août 1990
Accepté le 13 mars 1991

Life history characteristics of fish populations are influenced, genotypically and phenotypically, by the environmental characteristics of their habitat (Constantz 1979; Mann et al. 1984; Stearns and Crandall 1984). Thus, fish populations may exhibit differences in age and size at maturity, fecundity, and/or energy allocated to gonads according to latitude of the water body (Schaffer and Elson 1975; Leggett and Carscadden 1978; Mann et al. 1984), temperature (Alm 1959; Mills 1988), and food resources (Leggett and Power 1969; Deacon and Keast 1987; Trippel and Harvey 1989). Life history traits may also differ between fish populations subjected to varying degrees of environmental fluctuation. For example, a high degree of fluctuation in water level and/or flow was associated with large brood size and small size at maturity in tule perch (*Hysterocarpus traski*) (Baltz and Moyle 1982) and more energy allocated to reproduction in Gila topminnows (*Poeciliopsis occidentalis*) (Constantz 1979) and threespine sticklebacks (*Gasterosteus aculeatus*) (Crivelli and Britton 1987).

Dissolved oxygen levels also fluctuate in water bodies, and many shallow, north temperate lakes and ponds are subject to winter oxygen depletion. Fish inhabiting such environments may show physiological adaptations to hypoxia (e.g. crucian carp (*Carassius carassius*) (Holopainen and Hyvarinen 1985) or behavioral adaptations (e.g. central mudminnow (*Umbra limi*)) (Klinger et al. 1982). Fish that do not exhibit such adaptations (e.g. yellow perch (*Perca flavescens*)) are subject to high mortality when severe hypoxia occurs (Tonn and Paszkowski 1986).

Populations subjected to the recurrent risk of winterkill would be expected to show life history adaptations similar to those exhibited under other types of environmental fluctuation, but this has not been studied in fish. In this paper, we investigate the implications of winterkill stress on the reproductive life history of the pumpkinseed (*Lepomis gibbosus*), a common north temperate species that inhabits a broad range of water bodies, including those that winterkill (see Tonn and Magnuson 1982; Hall and Ehlinger 1989; Keast and Fox 1990). We predicted that populations inhabiting water bodies with winterkill risk would show precocial maturity and high reproductive invest-

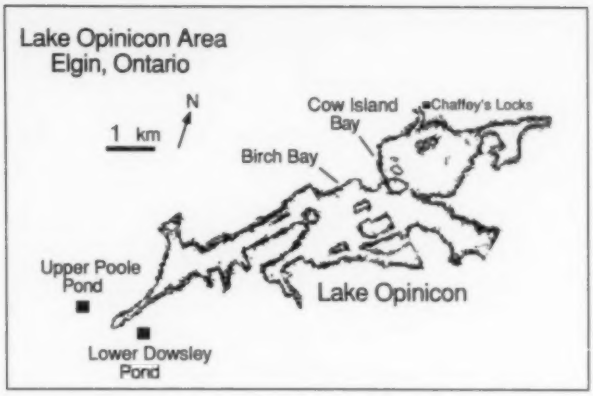

FIG. 1. Location of Cow Island Bay and Birch Bay collection sites in Lake Opinicon, and location of Upper Poole and Lower Dowsley ponds relative to the lake.

ment relative to those inhabiting stable, nonwinterkill environments. We also predicted that postwinterkill populations would differ in reproductive life history traits from those exhibited by the same populations in nonwinterkill years.

Methods

Study Sites

Three water bodies were used in the study: two beaver ponds subject to winterkill and an adjacent lake for comparison. All are located in Leeds County, Ontario (50 km north of Kingston), and are within 5 km of each other (Fig. 1).

Lower Dowsley Pond (area 2.6 ha) is the largest of a series of beaver impoundments along a stream channel. This pond is isolated from downstream waters by an outlet dam and a 2-m descent and from upstream waters by 3- to 10-m descents. Three fish species occur in Lower Dowsley: pumpkinseeds, redbelly dace (*Phoxinus eos*), and a few Iowa darters (*Etheostoma exile*). Maximum pond depth was 1.8 m during the study, and summer pH ranged from 7.8 to 8.2

Upper Poole (area 0.7 ha) is a shallow pond connected to a chain of small upstream lakes by a creek that flows partly underground, with beaver dams at the pond inlet and outlet. Fifteen species of fish were found in Upper Poole, dominated by blacknose and blackchin shiners (*Notropis heterolepis* and *N. heterodon*), pumpkinseeds, and brown bullheads (*Ictalurus nebulosus*). Largemouth bass (*Micropterus salmoides*) caught in the pond were mostly young-of-year, but a few adults were observed with mask and snorkel. Maximum pond depth was 1.8 m in the summers of 1986 and 1987, but a breach in the outlet dam in the winter of 1987 dropped subsequent water levels by about 1 m. (The dam was repaired by beavers the following spring). Summer pH ranged from 7.8 to 8.4.

Two severe winterkill events occurred over the course of the study, one in each of the ponds. The Lower Dowsley winterkill (>95% mortality) occurred in 1986–87 (Fox and Keast 1990). Hypoxia was the likely cause, as the pond was completely frozen over from late November 1986 to mid-April 1987 and dissolved oxygen levels were below 2 ppm by January. No winterkill occurred the following year.

Upper Poole Pond did not completely freeze over in either winter of observation, but a severe winterkill (87–96% mortality of age 1 and older fish) nevertheless occurred in 1987–88 when a rift in the dam dropped the water level enough to freeze much of the pond to the bottom. Although winterkill conditions were not evident the previous year, annual mortality was estimated at 37% in age 1 pumpkinseeds and 52% in age 2 and older individuals (Fox and Keast 1990).

TABLE 1. Population density and age structure of pumpkinseeds in the study sites (data from Fox and Keast 1990).

Water body	Year	Density (no·100 m^{-2})	Age distribution (%) 1	2	3	4	>5
Lower Dowsley Pond	1986	159	83	9	5	<1	<1
	Winterkill						
	1987	34	53	41	6	<1	<1
	1988	38	N/A	58	37	5	<1
Upper Poole Pond	1986	57	40	58	2	<1	<1
	1987	69	65	16	16	3	<1
	Winterkill						
	1988	12	N/A	83	17	<1	<1
Lake Opinicon	1987	15	N/A	40	33	20	7
	1988	15	N/A	13	20	25	40

Lake Opinicon (area 787 ha) is a shallow (maximum depth 11 m), mesotrophic lake that is not subject to winterkill. The lake contains large populations of pumpkinseed and bluegill (*Lepomis macrochirus*), as well as 14 other littoral zone species (Keast 1970). Pumpkinseed growth rate in Lake Opinicon is typical of that in several other lakes in the area and considerably higher than that in local lakes known to have stunted populations (see Keast 1978; Complak 1982; Deacon and Keast 1987). Pumpkinseeds were collected from two littoral zone sites at the northeast end of the lake: Cow Island Bay and Birch Bay (Fig. 1). The characteristics of these sites are described in Keast (1965, 1978).

Pumpkinseed population density and age structure in Lake Opinicon, Lower Dowsley Pond, and Upper Poole Pond were characterized in Fox and Keast (1990) and are summarized in Table 1. The two pond populations were subject to drastic fluctuations as a result of winterkills. Pond population structure was heavily concentrated towards the youngest age-classes. These pumpkinseeds rarely survived to age 5 whereas conspecifics in Lake Opinicon lived to age 8 and older.

Although Lake Opinicon was the only nonwinterkilll water body sampled in this study, pumpkinseed life history was previously studied in nearby Upper and Lower Beverly lakes (area 551 and 766 ha, respectively) by Deacon and Keast (1987). These nonwinterkill lakes, located 10–15 km from the study area, are connected to Lake Opinicon through the Rideau Canal system. Length at age data from Deacon and Keast (1987) suggested that typical pumpkinseed growth rates in Lake Opinicon and the Beverly lakes are equivalent up to age 4, after which time, growth in the Lower Beverly population is stunted relative to the other populations. We used Beverly lakes data in qualitative comparisons of age and size at maturity between winterkill and nonwinterkill populations, but we did not include these data in statistical tests.

Determination of Reproductive Life History Characteristics

Reproductive life history data were obtained from pumpkinseeds netted from Lower Dowsley and Upper Poole ponds in

1987 and from all three water bodies in 1988. Pond pumpkinseeds were collected with beach seines and funnel traps from May 19 to 27, prior to the onset of nesting activity in these sites. Collection of Lake Opinicon specimens began about a week later, as cooler lake temperatures delayed reproduction relative to the ponds. One batch of pumpkinseeds collected from the lake on June 6 could have spawned prior to capture and were therefore not used in gonadosomatic index and fecundity comparisons.

Total length and wet weight were measured on fresh specimens, and age was determined by reading acetate impressions of scales removed from the area around the tip of the pectoral fin. Annuli were identified using the criteria outlined in Regier (1962). Ages reported are those of the year of collection, even if the current year annulus had not yet formed.

Pumpkinseed gonads were inspected to determine sex and maturity status, and those of mature individuals were removed and weighed (fresh). Gonadosomatic index (GSI) means (Bagenal and Braum 1978) were calculated by water body, year, and age-class.

We estimated pumpkinseed fecundity in 1988, using a volumetric method similar to that described in Deacon and Keast (1987). Ovaries of mature females were split, placed in bottles containing Gilson's fluid, and shaken regularly to separate eggs from ovarian tissue. Eggs that remained attached to ovarian walls after 2 mo were manually separated, and the wall tissue was removed from the sample. Eggs were placed in a multisided Vertis flask, and the volume was brought to 100 mL with water. The mixture was mechanically stirred, and five subsamples of 5 mL each were removed with an Eppendorf pipette. Eggs that were opaque and had yolk visible were counted under a dissecting microscope. This included eggs in several size-classes ranging upward from 0.3 mm in diameter, considered to be those which a female could have spawned during the year of sampling (Deacon and Keast 1987). Total fecundity was calculated from the mean number of eggs in each subsample.

Statistical Analysis

Pumpkinseeds were classified by age and length in order to determine whether age or size at maturity was significantly different among the populations. The proportions of mature fish in 1988 were compared with G-tests, performed on arcsine-transformed data (Zar 1984). Maturity comparisons between pre- and postwinterkill years were made with G-tests on the number of mature and immature specimens in a "pivotal" age and size-class, where pivotal is defined as the earliest class that achieved 50% maturity in a given year.

To test whether populations had equal fecundity, we examined the $\log_e$-transformed body weight – fecundity relationships of these populations with analysis of covariance (ANCOVA) after first testing for homogeneity of slopes. GSIs of the populations were compared with Mann–Whitney U tests. The level of significance for all statistical tests was set at $P = 0.05$.

Results

Length at Age

Pumpkinseeds in the three water bodies displayed distinctive growth patterns (Fig. 2a). Upper Dowsley fish were the largest after age 2. Lower Poole pumpkinseeds showed better growth up to age 3 than did those of Lake Opinicon, but the two populations were nearly equivalent by age 4. Males were generally larger than females in all three populations.

Growth pattern differences in 1988 appeared to be representative of long-term trends (Fig. 2b). However, 1987 and 1988 growth rates differed in the two pond populations. Age 2 Lower Dowlsey pumpkinseeds sampled in May 1988 averaged 7 mm larger than their age equivalents in 1987, due to the high growth rate of the 1986–87 winterkill survivors (Table 2). These survivors showed rapid somatic growth even prior to reproduction. In contrast, Upper Poole fish caught in May showed little difference in length at age between pre- and postwinterkill years. Somatic growth in this population was higher following the winterkill, but the difference between years did not emerge prior to reproduction (Table 2).

Age at Maturity

Pumpkinseeds in both beaver ponds showed strong evidence of precocial maturity when compared with the Lake Opinicon and Beverly lakes populations (Fig. 3). This pattern was evident in both sexes, with approximately 40% of the age 2 females from both ponds and 40% of the Upper Poole age 2 males maturing in 1988. Nearly all pond pumpkinseeds were mature by age 3. In contrast, few females from Lake Opinicon or the Beverly lakes were mature until age 4, and few males were reproductive until age 5. The proportion of sexually mature fish by age-class was significantly different between Lake Opinicon and the two ponds (G-test, males and females combined, $P < 0.001$) whereas there was no significant difference between the two pond populations in 1988 ($P = 0.06$).

Beaver pond populations showed a shift towards earlier maturation following winterkill. In Lower Dowsley, 82% of the population matured at age 2 after the 1986–87 winterkill whereas only 25% matured at age 2 in 1988 (Table 3). This difference was significant for both males and females (G-test, $P < 0.001$ for males and $P = 0.002$ for females). A similar shift in the proportion of early (age 3) matures was evident in Upper Poole following the 1987–88 winterkill; however, the difference was significant in males (G-test, $P = 0.005$) but not females ($P = 0.50$).

Length at Maturity

Beaver pond populations became reproductive at a smaller size than their lake conspecifics (Fig. 4), and the difference in proportion of mature fish by length-class was significant in 1988 (G-test, males and females combined, $P < 0.001$). Lake–pond differences were most pronounced in males, with all pond males mature at 100 mm total length (TL), but less than half of the Lake Opinicon males were mature even at 120 mm. Less than half of the pumpkinseeds were mature at 100 mm in the slower growing population of Lower Beverly Lake, and no pumpkinseeds under 125 mm were mature in Upper Beverly Lake. Females tended to mature at a smaller size than males, particularly in the Lake Opinicon population (Fig. 4).

Lower Dowsley pumpkinseeds matured at a smaller size after the 1986–87 winterkill than in the succeeding year. The smallest reproductive female caught in 1987 was 65 mm TL whereas in 1988, the smallest reproductive female was 74 mm. The smallest mature male was 72 mm in 1987 versus 85 mm in 1988. More than 75% of the 70–79 mm pumpkinseeds sampled from Dowsley were mature in 1987 whereas only 1 of 25 males and 12 of 32 females under 100 mm were mature in the following year. In small (<80 mm) Dowsley pumpkinseeds, per-

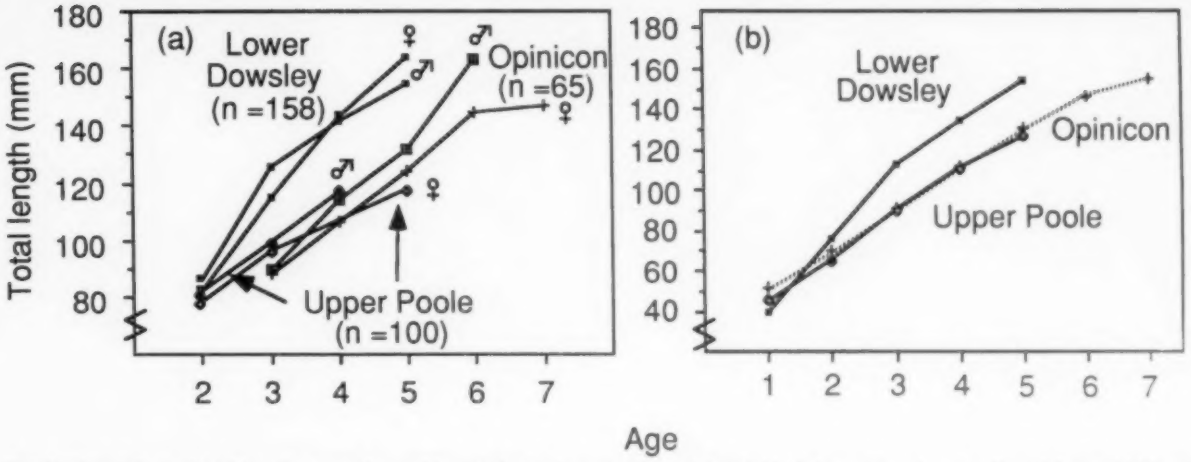

FIG. 2. Comparison of total length at age of pumpkinseeds in the three study sites (a) Individuals by sex; specimens collected from mid-May to early June 1988. (b) Mean backcalculated length of both sexes combined, based on specimens collected from May 1987 to August 1988; from Fox and Keast (1990).

TABLE 2. Length at age (mean ± 1 SE) and early spring growth of beaver pond pumpkinseeds prior to reproduction for pivotal ages, 1987 and 1988.

Pond	Year	*N*	Age	Mean length at annulus formation (mm)	Mean length at capture (mm)	Early season growth (mm)
Lower Dowsley	1987[a]	24	2	67.4 (1.3)	76.9 (1.3)	9.5
	1988	74	2	81.5 (0.7)	83.7 (0.8)	2.2
Upper Poole	1987	16	3	91.5 (1.8)	99.1 (1.7)	7.6
	1988[a]	34	3	93.9 (0.8)	99.4 (0.7)	5.5

[a]Postwinterkill years.

cent maturity in 1987 was significantly higher than in 1988 for both males (*G*-test, $P = 0.024$) and females ($P = 0.006$).

Upper Poole pumpkinseeds showed the same tendency to mature at a smaller size following winterkill. The between-year difference is highly significant in 90–110 mm males, 40% of which were mature in the prewinterkill year, compared with 90% in the postwinterkill year (*G*-test, $P = 0.002$). Postwinterkill females matured at lengths as small as 73 mm, and more than half of the 80–89 mm length-class were reproductive. Only two females <90 mm were netted in the previous year, so the between-year difference in length at maturity in Upper Poole females could not be tested.

Gonadosomatic Index and Fecundity

Beaver pond females not only matured earlier than Lake Opinicon females, but they also had higher gonadal weight and fecundity (Fig. 5 and 6). Ovaries of mature beaver pond females were typically 10% of body weight whereas those of females from Lake Opinicon and Upper and Lower Beverly lakes were generally less than 5% of body weight.

There were significant differences among the pond and Lake Opinicon populations in fecundity relative to body weight (ANCOVA, $P < 0.001$). Females in both ponds had significantly higher fecundities than Lake Opinicon females (ANCOVAs, $P < 0.001$). In addition, Upper Poole females were significantly more fecund than Lower Dowsley females in 1988 ($P = 0.02$), the year following the winterkill in Poole.

Mean testes weight of mature pumpkinseeds varied from 0.7% of body weight in Lower Beverly males to 1.4% in Upper Poole males. Both beaver pond populations had significantly higher GSIs than the Lake Opinicon population (Mann–Whitney *U* tests, $P < 0.05$).

Discussion

Variation between Winterkill and Nonwinterkill Populations

Beaver pond pumpkinseed populations subjected to winterkill conditions displayed a different reproductive life history pattern from those of the nonwinterkill lakes, including precocial maturity, smaller length at maturity, higher gonadal investment, and higher fecundity. These reproductive characteristics are typical of organisms living in environments favoring rapid population growth. Such "*r*-selected" characteristics (MacArthur and Wilson 1967) should be favored in habitats that experience conditions extreme enough to cause catastrophic mortality, followed by recolonization by the survivors. The maturation characteristics of beaver pond pumpkinseeds, along with their short life span (Fox and Keast 1990), are also consistent with the predictions of "bet-hedging" life history models for populations experiencing variable adult mortality in fluctuating environments (Schaffer 1974; Stearns 1976). These models show that such populations should breed early and maximize reproductive investment to avoid the risk of early mortality and thus, reduced contribution of offspring to the next generation.

While our study shows that populations inhabiting winterkill and non-winterkill environments have different life history patterns, our data do not eliminate other environmental factors as contributors to early maturation and high reproductive investment in pond populations. The beaver ponds used in our study are small and shallow and experience greater fluctuations in temperature, water level, and prey availability than a typical

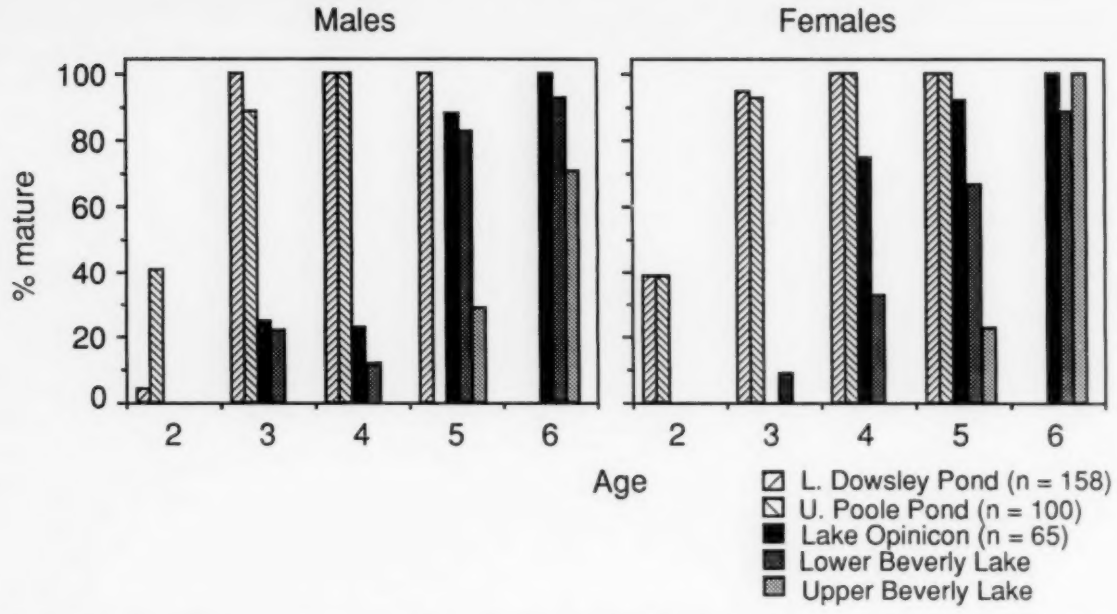

FIG. 3. Proportion of mature pumpkinseeds by age in the three study sites and two nonwinterkill lakes in the same watershed. Data on Upper and Lower Beverly Lakes are taken from Deacon and Keast (1987).

TABLE 3. Comparison of sexual maturity at ages 2 and 3 in Lower Dowsley and Upper Poole ponds, respectively, during winterkill and nonwinterkill years.

	No. of males		No. of females		Total	
Pond	Mature	Immature	Mature	Immature	Mature	Immature
Lower Dowsley (age 2)						
1987[a]	9	1	19	5	28	6
1988	1	25	14	22	15	47
Upper Poole (age 3)						
1987	5	7	5	1	10	8
1988[a]	16	2	14	1	30	3

[a]Postwinterkill years.

lake (Fox and Keast 1990). These factors contribute to environmental variability independently of winterkill and might therefore affect reproductive life history traits of pumpkinseeds even if winterkill did not occur. However, the between-year comparisons in our study show clearly that winterkill has a strong influence on maturation, and populations subjected to the recurring risk of winterkill would undoubtedly experience stronger selective pressures than those living in less extreme environments.

Pumpkinseeds inhabiting these winterkill ponds showed life history adaptations consistent with those of other fish populations living in variable and/or unpredictable habitats. Constantz (1979) found that Gila topminnows inhabiting a fluctuating desert wash in Arizona were more fecund and allocated more energy to reproductive tissue than conspecifics living in a spring with nearly constant flow. Baltz and Moyle (1982) showed that tule perch length at maturity and life span decreased, and mean brood size increased, with increasing water level fluctuation in four California populations. Schmeidler and Brown (1990) found that plains killifish (*Fundulus zebrinus*) in two Kansas rivers with variable flow regimes had higher fecundities relative to body weight than killifish inhabiting a more stable river in the basin. Threespine sticklebacks inhabiting a wetland complex on the Mediterranean coast subject to high seasonal water level fluctuations were shown by Crivelli and Britton (1987) to exhibit much higher fecundity than other European populations living in more stable environments. These authors also noted a large difference in length at maturity of sticklebacks collected in two succeeding years, but no other between-year differences were documented in this semelparous population.

Early reproduction, high gonadal investment, and high fecundity can also be favored as a result of abundant food resources and/or rapid somatic growth in some fish species (Alm 1959; Constantz 1979; Mann et al. 1984; Mills 1988). However, populations of other species that grow rapidly and have abundant food resources show opposing life history traits such as delayed maturity, low gonadal investment, and/or low fecundity (Leggett and Power 1969; Pinhorn 1969; Deacon and Keast 1987; Trippel and Harvey 1987). This apparent contradiction has been explained in the literature by genetic differences in stocks being compared (Alm 1959), change in

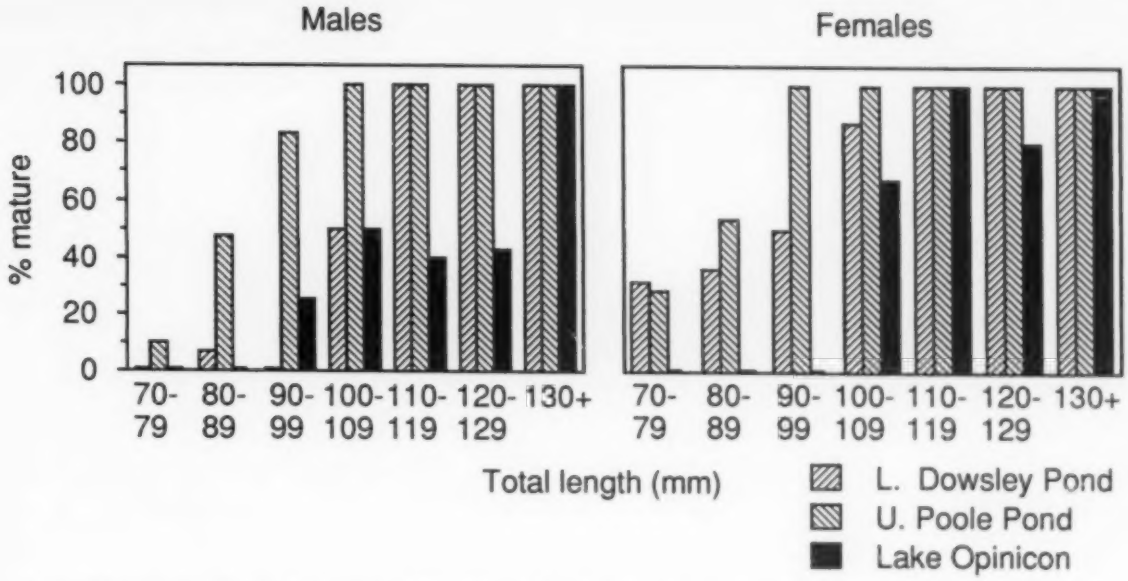

FIG. 4. Proportion of mature pumpkinseeds by total length in the three study sites; 1988 data. Sample sizes are given in Fig. 3.

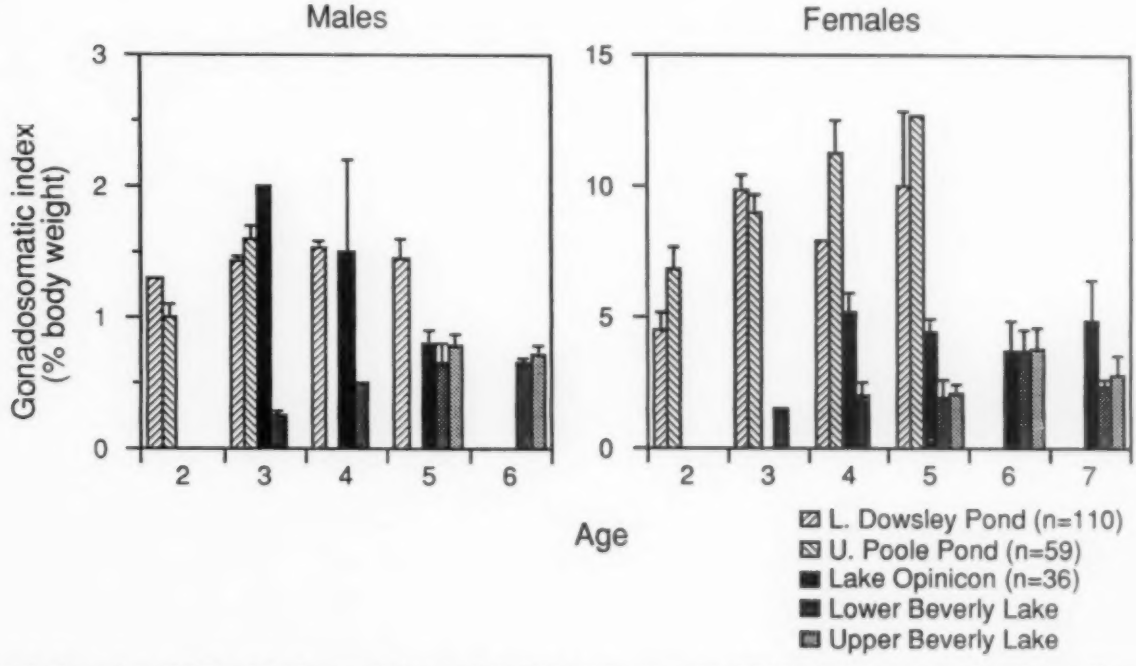

FIG. 5. Comparison of GSI's of the three populations studied and those of two nonwinterkill lakes in the same watershed. Data on Upper and Lower Beverly lakes are taken from Deacon and Keast (1987). Vertical bars represent ± 1 SE.

the relative growth characteristics of stocks prior to and subsequent to maturity (Alm 1959), and the effects of water temperature and life span on reproductive life history traits (Wootton 1977; Mann et al. 1984; Mills 1988). In addition, the rate of mortality may be important in the evolution of life history parameters in fish (Roff 1984), and mortality can be independent of growth (Stearns and Crandall 1984).

Differences in growth characteristics alone are not likely to be the sole cause of life history differences among the three populations studied. For example, Deacon and Keast (1987) showed that earlier maturity and higher reproductive investment occurred in the Lower Beverly Lake pumpkinseed population, despite its slower growth rate relative to the Upper Beverly Lake population. However, the slower growing population had

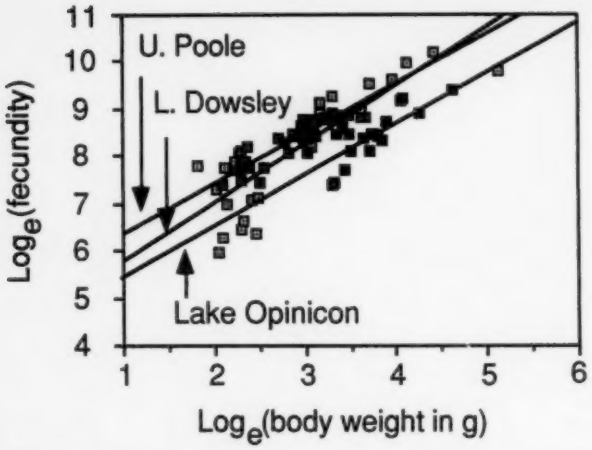

FIG. 6. Relationship of fecundity to body weight in pumpkinseed females of Lower Dowsley Pond (⊡), Upper Poole Pond (⊞), and Lake Opinicon (■) in 1988. Data are $\log_e$-transformed.

a higher adult mortality rate (Deacon and Keast 1987), and this would favor the evolution of earlier maturation (see Roff 1984).

Growth and mortality in fish can be affected unequally by environmental factors such as resource availability (Hall et al. 1970; Fox 1989) and degree of environmental fluctuation (Fox and Keast 1990). Thus, resource availability and environmental fluctuation may act as independent selection factors affecting reproductive life history traits such as the timing of maturation and reproductive investment. Taylor et al. (1990) proposed a model for vegetation that separates the influence of resource impoverishment and environmental disturbance on characteristics such as relative energy allocation to somatic and reproductive functions. This "habitat templet" model leads to predictions of increased reproductive allocation with increases in either resource availability or degree of environmental disturbance. The Taylor et al. (1990) model is consistent with lake–pond differences in pumpkinseed gonad weight and fecundity determined in our study. It could also account for high energy allocation into reproduction of a slow-growing population, but only if this population has been subjected to a high degree of environmental disturbance.

In our study, both environmental fluctuation and rapid growth appeared to be acting in concordance to produce the distinctive reproductive life history pattern of the winterkill populations. Despite higher prey density in the lake, pumpkinseed growth in the two beaver ponds was as good as or better than that of the lake population as a result of population density reduction following winterkill and the dearth of interspecific competitors in these ponds (Fox and Keast 1990). In addition, pond temperatures were higher than lake temperatures immediately prior to reproduction, a time when favorable conditions would likely promote allocation of energy into gonads (see Constantz 1979; Diana 1983; Trippel and Harvey 1987).

Finally, there is good evidence that winterkills are a frequent occurrence in these ponds. In Lower Dowsley, a second winterkill in 4 yr occurred in the winter of 1989–90. In Upper Poole, aerial photographs over the last two decades showed that the pond was much smaller and shallower in the recent past (Keast and Fox 1990), and thus was more likely to become hypoxic in winter. The occurrence of recurrent winter hypoxia in an isolated water body would make delayed reproduction a high-risk strategy in pumpkinseeds, since this species has no special ability to tolerate low oxygen concentration (see Moore 1942).

Changes in Reproductive Life History following Winterkill

Pumpkinseeds in both ponds matured earlier and at a smaller size in winterkill years than they did in nonwinterkill years. These annual differences were consistent with those exhibited between winterkill and nonwinterkill populations. The fact that such changes could be manifested by populations in successive years suggests a high degree of phenotypic plasticity in the reproductive life history pattern of this species. Previous experiments by Alm (1959) with Eurasian perch (*Perca fluviatilis*), brown trout (*Salmo trutta*), and other species stocked in artificial ponds suggested that this was the case, but our study shows that this plasticity can be expressed rapidly under natural conditions. However, our study does not show that the difference among populations is due solely to environmental factors. The suite of life history traits has a genetic basis (Alm 1959; McPhail 1977; Stearns and Crandall 1984; Trexler and Travis 1990). Unfortunately, few fish studies have examined the relative importance of genetic and environmental factors in the expression of these traits.

One final question raised by our study is what information is used by pumpkinseeds to determine whether or not to mature and how much energy to put into reproduction. In the case of a population emerging from winterkill conditions, the ability of survivors to obtain abundant energy supplies prior to reproduction could be the major factor (see Constantz 1979). Alternatively, the dearth of large conspecifics in the environment could indicate that an investment in reproduction is potentially profitable. Information on the abundance of larger conspecifics could be especially important to an individual if social interactions are capable of preventing maturation in pumpkinseeds. Social inhibition of maturation is known to occur in some short-lived fishes (Sohn 1977; Borowsky 1978; Jones and Thompson 1980).

In summary, our study shows that pumpkinseed populations living in water bodies subject to winterkill exhibit life history trends consistent with those of fishes living in other fluctuating habitats. Pumpkinseed populations respond to winterkill by advancing maturity and increasing reproductive investment. A high degree of phenotypic plasticity in life history traits is indicated, but further research is required to determine the type of information used by these fish to alter the average reproductive investment after a winterkill event.

Acknowledgements

Thanks are due to Ian Smith and Christine Watters for their assistance in the field and laboratory and to Raleigh Robertson, Frank Phelan, and Floyd Connor for the use of facilities at the Queen's University Biological Station. We also appreciate the helpful comments on a previous version of this manuscript provided by Tim Ehlinger. Financial support for this study was provided by the Natural Sciences and Engineering Research Council of Canada through a grant to A. Keast. M. Fox was supported by a McLaughlin Fellowship while at Queen's University.

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Biochemical Characterization of Third-Stage Larval Sealworm, *Pseudoterranova decipiens* (Nematoda:Anisakidae), in Canadian Atlantic Waters using Isoelectric Focusing of Soluble Proteins

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Appleton,T. E., and M. D. B. Burt. 1991. Biochemical characterization of third-stage larval sealworm, *Pseudoterranova decipiens* (Nematoda:Anisakidae), in Canadian Atlantic waters using isoelectric focusing of soluble proteins. Can. J. Fish. Aquat. Sci. 48: 1800–1803.

Isoelectric focusing (IEF) was performed on soluble protein extracts from whole specimens of third-stage larval sealworm (*Pseudoterranova decipiens*) recovered from the musculature of three fish intermediate host species: Atlantic cod (*Gadus morhua*), sea raven (*Hemitripterus americanus*), and rainbow smelt (*Osmerus mordax*). The fish were collected at various sites in the Canadian Atlantic, and IEF revealed the occurrence of two "variants" within what has previously been considered a single, uniform species of *P. decipiens* in these waters. The larvae were characterized by the absence ("type I" L_3's) or presence ("type II" L_3's) of a sharp, dark-staining protein band with a mean pI of 6.46 pH units. Type I larvae were predominant at two sites sampled in the lower Bay of Fundy, while type II larvae were predominant at three sites sampled in the Gulf of St. Lawrence region.

On a pratiqué l'électrofocalisation sur des extraits protéiniques solubles de sujets entiers du ver de phoque (*Pseudoterranova decipiens*) au troisième stade larvaire, qui ont été prélevés dans les muscles de sujets de trois espèces d'hôtes intermédiaires, soit la morue de l'Atlantique (*Gadus morhua*), le hémitriptère atlantique (*Hemitripterus americanus*) et l'éperlan arc-en-ciel (*Osmerus mordax*). Les poissons ont été prélevés en différents points de la région atlantique du Canada; l'électrofocalisation a révélé l'existence de deux « formes variantes » qui composent ce que l'on croyait être une espèce unique et uniforme de *P. decipiens* dans ces eaux. Les larves ont été caractérisées selon l'absence (L_3 de « type I ») ou la présence (L_3 de « type II ») d'une bande sombre et nette dont le point isoélectrique moyen est de 6,46 unités de pH. Les larves du type I étaient prédominantes à deux stations d'échantillonnage situées dans le fond de la baie de Fundy, alors que les larves du type II étaient prédominantes à trois stations d'échantillonnage situées dans la région du golfe du Saint-Laurent.

Received August 9, 1990
Accepted April 10, 1991
(JA675)

Reçu le 9 août 1990
Accepté le 10 avril 1991

The frequent occurrence of the third-stage larvae (L_3's) of sealworm (*Pseudoterranova decipiens*) in the musculature of commercially important fishes, particularly Atlantic cod (*Gadus morhua*), is a chronic cosmetic problem and one that costs the commercial fisheries in Atlantic Canada annual estimates of $26.6 million (Malouf 1986).

Efforts to find a means of controlling sealworm abundance in marketable fish have included the organization of two international workshops by the Department of Fisheries and Oceans (Canada) (at the instigation of Dr. J. E. Stewart, Bedford Institute of Oceanography, Dartmouth, N.S.) with the aim of developing a predictive model of the population dynamics of the worm (Bowen 1990). Detailed knowledge of the life cycle of this parasite is essential to the development of such a model and, in view of this, it is of the utmost importance to establish if the sealworms present in Canadian Atlantic waters represent a uniform species with a single life cycle or a complex of intraspecific variants or sibling species which may have different life cycles (McClelland et al. 1990).

Studies performed over the last 20–30 yr indicate that intraspecific variation in parasites is a widespread phenomenon and that many parasites can no longer be regarded as uniform species (Thompson 1982). In certain groups of Nematoda, such as the superfamily Ascaridoidea to which *P. decipiens* belongs, taxonomy at the level of genera and below is difficult due to the occurrence of convergence and parallelism resulting from an endoparasitic existence (Paggi et al. 1985). Thus, taxonomists have investigated alternative means of nematode diagnosis such as electrophoresis and isoelectric focusing (IEF) to examine soluble protein profiles and several specific enzyme loci.

Studies performed over the last 12 yr, using electrophoresis to examine a high number of enzyme loci, indicate that the occurrence of sibling species (species that are reproductively isolated but morphologically almost identical) in the Ascaridoidea is widespread. In the marine Ascaridoidea, *Anisakis simplex* consists of two sibling species (Nascetti et al. 1983), *Contracaecum rudolphi* consists of three sibling species, and *C. osculatum* consists of two sibling species (Berland et al. 1987). *Pseudoterranova decipiens* has been found to comprise three sibling species, designated A, B, and C (Matteucci and Paggi 1989). In the Canadian Atlantic, *P. decipiens* A appears to be absent and sibling B occurs in both harbour seals (*Phoca vitulina*) and grey seals (*Halichoerus gyrpus*). Sibling C has been found only in Atlantic cod from northern Labrador and in

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the bearded seal (*Erinrathus barbatus*) (McClelland et al. 1990), a species which ranges north of Newfoundland (Banfield 1974).

Despite the reported presence of only *P. decipiens* B in the Canadian Atlantic by Paggi et al. (1990), there are reasons for suspecting heterogeneity within the species in Canadian Atlantic waters: there is apparent low host specificity with respect to the definitive seal hosts (Margolis and Arai 1989) and to invertebrate and fish intermediate hosts (McClelland et al. 1990). Hence, we undertook an investigation of soluble protein profiles generated by IEF of soluble protein extracts from third-stage larvae of *P. decipiens* collected from different fish hosts and from different geographical locations in Canadian Atlantic waters.

Materials and Methods

Third-stage larvae of *P. decipiens* were dissected from the musculature of three species of fishes sampled from five sites in Canadian Atlantic waters. Sixty larvae from Atlantic cod from Passamaquoddy Bay, N.B. (approximately 45.05N, 66.00W), were compared with 10 larvae from sea ravens (*Hemitripterus americanus*) from Passamaquoddy Bay, 20 larvae from sea ravens and 40 larvae from Atlantic cod from just off the southeastern shore of Grand Manan Island, N.B. (approximately 44.40N, 66.50W), 42 larvae from Atlantic cod sampled from commercial catches from Chaleur Bay, N.B. (approximately 48.00N, 65.00W), 41 larvae from Atlantic cod sampled from commercial catches from the Gulf of St. Lawrence, and 22 larvae from rainbow smelt (*Osmerus mordax*) sampled from the Miramichi River and from Miramichi Bay, N.B. (approximately 47.05N, 65.10W). Except for fish sampled from commercial catches, Atlantic cod and sea ravens were caught by otter trawl. Rainbow smelt were caught by seining.

The worms were washed several times in 0.01 M phosphate-buffered saline (PBS) (pH 7.2) containing a detergent, "Tween," and then rinsed twice in 0.01 M PBS (pH 7.2). They were briefly blotted, weighed to the nearest milligram, and stored at −80°C.

Prior to running a gel, the samples were thawed on ice and then homogenized in a Pyrex tissue grinder with the addition of 200 μL of rinsing buffer for every 10 mg of worm tissue. The homogenate was centrifuged twice for 15 min at 15 000 × *g* at 4°C. Between spins, the supernatant was drawn off and the pellet was discarded. The protein concentration of each sample was determined using Bradford's (1976) assay (Bio-Rad).

Low-percentage polyacrylamide gels, 2 mm in thickness and with a broad pH range of 3.5–9.5, were prepared as described by Dixon and Arai (1985). Samples were focused using an LKB 2117 Multiphor II electrophoresis unit and a Fisher Biotech FB500 power supply. The gel template was cooled using a Neslab RTE-110 refrigerated water circulator set at 4°C. The electrode strips were soaked in 1 M NaOH (cathode) and 1 M H_3PO_4 (anode) and placed on opposite ends of the gel. Twenty 2 × 10 mm filter paper application pieces (LKB) were positioned 5 mm from the cathode and 20 μL of lysate was applied to each. The power supply was set to deliver a constant power of 30 W. Sample application pieces were removed after 0.5 h and the run was resumed for a further 1 h to give a total focusing time of 1.5 h.

Following electrofocusing, 10-mm gel strips were cut from the gel every 5 mm from cathode to anode for use in preparation of gel pH gradient curves as described by Dixon and Arai (1985). The remainder of the gel was placed in fixative (34.6 g of sulphosalicylic acid, 115 g of trichloracetic acid in 1 L of distilled water) for 0.5 h, transferred to destaining solution (250 mL of 95% ethanol, 80 mL of acetic acid in 1 L of distilled water) for 0.5 h, and then stained (0.25 g of Coomassie Blue K250 in 200 mL of destaining solution heated to 60°C and filtered through Whatman No. 3 filter paper) for 10 min. Following staining, gels were rinsed in several changes of destaining solution until a clear background was obtained.

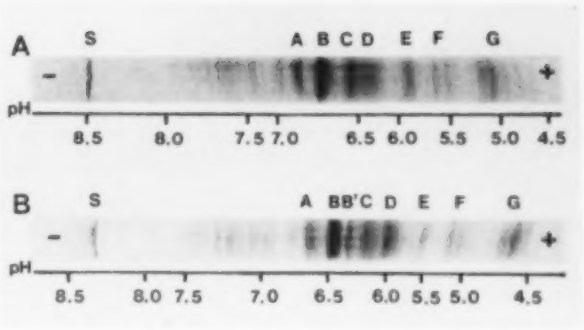

FIG. 1. Soluble protein profiles from polyacrylamide gel IEF of extracts from *P. decipiens* L_3's. (A) Type I; (B) type II.

Following destaining, the gels were photographed and each lane of each gel was scanned using a Hoefer GS300 scanning densitometer. Finally, the gels were preserved by drying onto a paper backing using a Bio-Rad model 483 slab dryer.

Results

Third-stage larvae weighed 0.024 (0.005–0.040) g, while protein concentrations of the lysates were 1.147 (0.307–2.540) mg/mL. Proteins focused predominantly in the pH range of 4.5–7.5 and protein profiles were not visibly altered by the variable protein concentrations of the lysates.

Of 60 L_3's from Atlantic cod from Passamaquoddy Bay, 57 (95.0%) had a consistent, characteristic protein profile as assessed by visual examination of gel lanes (Fig. 1A) and by examination of scanning densitometer traces (Fig. 2A). This protein profile was characterized by five dominant bands labelled A to E and two complexes of bands labelled F and G. Band A invariably consisted of a single, narrow protein band. Band B generally consisted of two closely packed bands that appeared as a single broad peak on densitometer traces. Similarly, bands C and D were each composed of two closely packed bands that generally appeared as single peaks on densitometer traces. In some cases, the presence of two narrow bands within these broad bands was apparent on densitometer traces as two peaks atop a broad, common base. Band E always consisted of a single, narrow band which varied in intensity. While the presence of the two complexes F and G was characteristic of these larvae, the degree of differentiation of the bands within each complex varied within the population and hence was not considered characteristic. Third-stage larvae exhibiting this profile were termed "type I" larvae.

The remaining 3 of 60 L_3's (5.0%) from Atlantic cod from Passamaquoddy Bay were characterized by a protein profile that differed from that of type I larvae by the presence of an

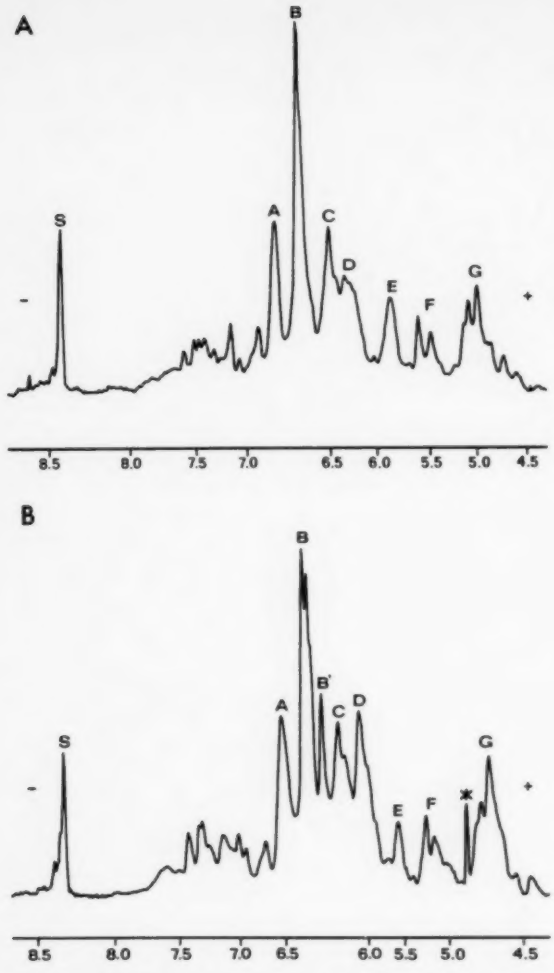

FIG. 2. Scanning densitometer traces of polyacrylamide gel IEF of extracts from *P. decipiens* L_3's. (A) Type I; (B) type II.

extra dark-staining band, labelled B′, between bands B and C (Fig. 1B and 2B) and the frequent presence of three, rather than two, closely packed, narrow bands within the B band itself. In some cases, however, band B was composed of either a single narrow band or, rarely, two closely packed, narrow bands. Bands A, C, D, and E and the complexes F and G were identical to those characteristic of type I larvae. Larvae characterized by the presence of the B′ band were termed "type II" larvae.

Both types of L_3 occurred at all sites sampled (Table 1). In fish from the two sites sampled in the lower Bay of Fundy, Passamaquoddy Bay, and near Grand Manan Island, larvae were primarily type I, although type II larvae were more common in sea ravens than in Atlantic cod at these sampling sites. In fish from sites sampled in the Gulf of St. Lawrence, Miramichi Bay, and Chaleur Bay, larvae were primarily type II. Both type I and type II larvae occurred in all three fish species sampled and, in some cases, occurred within the same host individual. Both types of larvae were found among small, cream-coloured L_3's as well as among large, red–brown L_3's.

Discussion

Third-stage larvae of *P. decipiens* in Canadian Atlantic waters are characterized by two distinct soluble protein profiles that differ by the absence (type I L_3's) of presence (type II L_3's) of a sharp, dark-staining protein band with a mean isoelectric point of 6.46. The identity and function of the unique protein band are unknown but the intensity of staining of the band suggests that, when present, the protein occurs in significant concentrations. That the unique protein band does not represent fish host material, either deposited or ingested, is supported by the observation that both type I and type II L_3's were found in all three fish host species examined and that there were no differences in protein profiles of worms occurring in different host species.

Based on the geographic distribution of the two types of L_3's, we speculate that the variation may be due to geographic distance between different populations, resulting in limited gene flow between them. Both the fish intermediate hosts (Scott and Scott 1988) and the seal definitive hosts (Stobo et al. 1990) of the sealworm are mobile and potentially capable of long migrations. Even so, our evidence suggests that the sealworms that occur in the lower Bay of Fundy (mainly type I L_3's) and those that occur in the Gulf of St. Lawrence are (mainly type II L_3's) represent somewhat distinct populations between which only limited gene flow might occur.

While the intraspecific variation is most likely a geographic phenomenon, there is some circumstantial evidence to suggest that the different variants mature and reproduce preferentially in different definitive hosts. Infectivity studies performed in this laboratory by Dr. L. Jarecka found that there were inconsistencies in the infectivities of L_2's, from different sources, to gammarids. The L_2's from eggs dissected from gravid female worms from grey seals were directly infective to gammarids and did not need to be ingested within a copepod paratenic host. On the other hand, L_2's from eggs dissected from gravid female worms from harbour seals were not directly infective to gammarids and did require being first eaten by a copepod. This

TABLE 1. Number and percentage of *P. decipiens* type I and type II larvae dissected from three species of fish caught at various sites in Canadian Atlantic waters.

Geographic site	Host	Number (%) of type I L_3's	Number (%) of type II L_3's
Passamaquoddy Bay, N.B.	*Gadus morhua*	57 (95)	3 (5)
Passamaquoddy Bay, N.B.	*Hemitripterus americanus*	5 (50)	5 (50)
Grand Manan, N.B.	*Gadus morhua*	34 (85)	6 (15)
Grand Manan, N.B.	*Hemitripterus americanus*	15 (75)	5 (25)
Miramichi Bay, N.B.	*Osmerus mordax*	9 (41)	13 (59)
Chaleur Bay	*Gadus morhua*	10 (24)	32 (76)
Gulf of St. Lawrence	*Gadus morhua*	14 (34)	27 (66)

latter finding indicates clearly that there is variation in the infectivity of certain worms at the early life cycle stages and strongly supports the conclusions of this investigation; differences in soluble protein profiles of L_3's indicate that there are two intraspecific variants of *P. decipiens* in the Gulf of St. Lawrence and more southerly Canadian Atlantic waters. Work in progress in this laboratory on variation in soluble protein profiles at the adult stage of the nematode may help to determine whether the variation is a geographical phenomenon or whether different variants occur preferentially in different definitive hosts.

Acknowledgements

We gratefully acknowledge Dr. Brent Dixon (Health and Welfare Canada) and Dr. Julie Dais (Grand Prairie Regional College) for assistance with IEF techniques. Mr. T. Landry (DFO, Moncton, N.B.) kindly provided smelt samples from Miramichi Bay, N.B. Huntsman Marine Science Centre staff assisted greatly in collection of Atlantic cod and sea raven samples. We thank Mr. T. Greaves for assistance in the field and Ms. C. Likely and Dr. L. Jarecka (Department of Biology, University of New Brunswick) for laboratory assistance and fruitful discussion. This research was supported by NSERC of Canada (Operating Grant A2358), a DFO Research Subvention Grant, a contract from the Federal/Provincial Subsidiary Agreement through the New Brunswick Department of Commerce and Technology to M.D.B.B., and by a New Brunswick Museum Florence M. Christie Research Scholarship to T.E.A.

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Tumor Frequencies in Walleye (*Stizostedion vitreum*) and Brown Bullhead (*Ictalurus nebulosus*) and Sediment Contaminants in Tributaries of the Laurentian Great Lakes

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Baumann, P. C., M. J. Mac, S. B. Smith, and J. C. Harshbarger. 1991. Tumor frequencies in walleye (*Stizostedion vitreum*) and brown bullhead (*Ictalurus nebulosus*) and sediment contaminants in tributaries of the Laurentian Great Lakes. Can. J. Fish. Aquat. Sci. 48: 1804–1810.

To better characterize neoplasm epizootics in the Great Lakes basin and their association with families of contaminants, we sampled five locations: the Fox and Menominee rivers, Lake Michigan; Munuscong Lake, St. Mary's River; and the Black and Cuyahoga rivers, Lake Erie. Frequencies of external and liver tumors were determined for brown bullhead (*Ictalurus nebulosus*) from all locations except the Black River and for walleye (*Stizostedion vitreum*) from the Lake Michigan and St. Mary's River sites. Sediment samples were analyzed for metals, polychlorinated aromatics, and polynuclear aromatic hydrocarbons (PAH). Liver neoplasms occurred in brown bullhead from the Cuyahoga River and Munuscong Lake; brown bullhead captured from Munuscong Lake were older than those collected from the other locations. Brown bullhead from these same two rivers had elevated hepatosomatic indexes. No liver neoplasms were found in brown bullhead from the Fox and Menominee rivers, although polychlorinated aromatics were highest in both Fox River sediment and Fox and Menominee brown bullhead, and arsenic was highest in Menominee River sediment and fish. Liver neoplasms in brown bullhead from the Cuyahoga River fit the prevailing hypothesis that elevated PAH in sediment can induce cancer in wild fish. The cause of the liver neoplasms in Munuscong Lake brown bullhead is undetermined.

Afin de mieux caractériser les épizooties à caractère néoplasique observées danas le bassin des Grands Lacs ainsi que leur association avec certaines familles de contaminants, nous avons procédé au prélèvement d'échantillons à cinq stations : les rivières Fox et Menominee, le lac Michigan, le lac Munuscong, la rivière St. Mary's ainsi que les rivières Black et Cuyahoga, lac Érié. On a déterminé la fréquence des tumeurs hépatiques et externes chez la barbotte (*Ictalurus nebulosus*) à tous les endroits sauf la rivière Black, ainsi que chez le doré (*Stizostedion vitreum*) des stations du lac Michigan et de la rivière St. Mary's. Les échantillons de sédiments ont été soumis à des dosages des métaux, des composés aromatiques polychlorés ainsi que des hydrocarbures aromatiques polynucléaires (HAP). Des néoplasmes du foie ont été observés chez la barbotte de la rivière Cuyahoga et du lac Munuscong; la barbotte capturée dans le lac Munuscong était plus vielle que celle capturée aux autres stations. La barbotte de ces deux mêmes rivières avait un indice hépatosomatique élevé. Aucun néoplasme hépatique n'a été observé chez la barbotte des rivières Fox et Menominee bien que la concentration en composés aromatiques polychlorés était la plus élevée dans les sédiments de la rivière Fox ainsi que dans la barbotte des rivières Menominee et Fox, et que la concentration de l'arsenic ait été la plus élevée dans les sédiments et le poisson de la rivière Menominee. L'observation de néoplasmes hépatiques chez la barbotte de la rivière Cuyahoga correspond à l'hypothèse dominante à l'effet qu'une concentration élevée d'HAP dans les sédiments peut conduire à l'apparition de cancers chez des populations sauvages de poisson. On ne sait pas pourquoi des néoplasmes hépatiques étaient observés dans la barbotte du lac Munuscong.

Received August 9, 1990
Accepted April 5, 1991
(JA676)

Reçu le 9 août 1990
Accepté le 5 avril 1991

Fishes from a variety of locations in North America have had elevated frequencies of liver neoplasia, including such species as English sole (*Parophrys vetulus*) from Puget Sound, Washington (Malins et al. 1984); Atlantic tomcod (*Microgadus tomcod*) from the Hudson River, New York (Smith et al. 1979); walleye (*Stizostedion vitreum*) and sauger (*Stizostedion canadense*) from Torch Lake, Michigan (Black et al. 1982); and winter flounder (*Pseudopleuronectes americanus*) from Boston Harbor, Massachusetts (Murchelano and

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Wolke 1985). Some tributaries and embayments of the Laurentian Great Lakes also contain populations of fish, primarily brown bullhead (*Ictalurus nebulosus*), in which epizootics of liver neoplasia have been documented. For instance, Baumann et al. (1987) found a 28–44% incidence of grossly observable liver neoplasia in brown bullhead collected from the Black River, Ohio, between 1980 and 1982.

At many of these locations the increased tumor incidence has been associated with elevated carcinogen levels in the sediment. Polynuclear aromatic compounds (PAH), in particular, have been hypothesized as causing liver cancer in wild populations of both English sole (Malins et al. 1984) and brown bullhead (Baumann et al. 1987).

One objective of the present work was to better define the extent of liver neoplasia in a benthic and a pelagic species widely distributed in the Great Lakes watershed. The second objective was to test the association of neoplasms with different classes of toxicants by choosing study sites characterized by different types of contaminants.

Methods

Site selection and methodology for collection and analysis of PAH in sediment at our study sites were described by Fabacher et al. (1988). We collected sediment for chemical analysis from five locations: the Fox and Menominee rivers, Lake Michigan; Munuscong Lake, St. Mary's River; and the Black and Cuyahoga rivers, Lake Erie. Munuscong Lake was included as a potential reference location because it did not have industrial point sources. The Black River was selected as a positive control because previous studies had documented the presence of sediment contaminants and their genotoxicity (Baumann et al. 1982; West et al. 1985; West et al. 1986) and the incidence of neoplasia in its brown bullhead population (Baumann et al. 1987; Baumann et al. 1990). A composite sediment sample of about 35 L was taken from selected depositional zones in each tributary (Fabacher et al. 1988) with a Ponar dredge (523 cm^2).

We selected walleye and brown bullhead as representative pelagic and benthic species, respectively. Fish were not collected from the Black River as part of this study (1984) because a large ($N = 125$) sample of brown bullhead livers from this location had been analyzed histopathologically in 1982 (Baumann et al. 1990). At the other four locations, we atempted to collect 50 fish of each species. Only walleye longer than 300 mm total length (TL) and brown bullhead longer than 200 mm TL were examined to ensure that the fish were 2 yr old or older because tumor incidence increases with age (Baumann et al. 1987). At two locations, not enough brown bullhead were available; thus, six of the 46 bullhead from the Menominee River and 21 of the 52 bullhead from the Fox River were black bullhead (*Ictalurus melas*); only brown bullhead were included in the random sample for histopathology and chemical analysis. We were unable to collect adequate samples of walleyes from the Cuyahoga River or waters near its mouth.

Fish were measured, weighed, examined for external tumors, and autopsied. Spines or scales were removed for aging. The liver was removed, weighed, and examined for grossly visible tumors. All suspected lesions and surrounding tissues were preserved in 10% neutral buffered formalin and subjected to histopathological analysis. The first 10 fish of each species were later frozen on dry ice for chemical analysis. The next 10 livers from each species that appeared to be normal under gross examination were also preserved. All preserved tissues were processed for histopathology at the Registry of Tumors in Lower Animals, Smithsonian Institution, and diagnoses of neoplasms were made by one of us (J.C.H.) Histopathological procedures and diagnostic criteria followed Baumann et al. (1990). In this manuscript the term "tumor" will be used to indicate cancers, other true neoplasms, and epidermal papillomas. Suspected preneoplasm lesions such as hepatocellular alteration and cholangiofibrosis (adenofibrosus) will not be included under the term tumor. Liver weight was divided by body weight for each fish to obtain a hepatosomatic index (HSI), which is also referred to as relative liver weight (Fabacher and Baumann 1985). Correlation coefficients between HSI and fish length, weight, liver weight, age, and collection date were determined for each species at each site. We also used two-way analysis of variance to examine the effects of site and sex on the HSI for both walleye and brown bullhead. Tukey's Studentized range test was used to contrast means where significant differences ($p = 0.05$) were detected. We used the Statistical Analysis Systems (SAS).

Polychlorinated dibenzo-*p*-dioxins, dibenzofurans, and ortho-non-substituted polychlorinated biphenyls (PCB) were analyzed in sediment and fish using the technique of Smith et al. (1984). Sediment and fish samples were prepared and analyzed for concentrations of selected elements by one of two methods: (1) samples were prepared for As with a magnesium nitrate dry ashing procedure (May 1982) and the elements were determined by hybride generation atomic absorption spectrophotometry; (2) samples were prepared for Be, Cd, Cr, and Ni analysis by subjecting them to a perchloric/hydrofluoric digestion procedure similar (with some modifications) to that described by Tessier et al. (1979) for the "residual metals" fraction. These four elements were determined by conventional flame atomic absorption spectrophotometry procedures. Values for sediment PAH were reported in Fabacher et al. (1988); PAH concentrations in fish tissue were determined by capillary gas chromatography and photoionization detection using the procedure of Zajicek (1987) as derived from Vassilaros et al. (1982). The term "carcinogenic" is applied here only to the PAH listed as having "sufficient evidence" of carcinogenicity to experimental animals by the International Agency for Research on Cancer, World Health Organization (1987).

Results

Hepatosomatic Indexes

The HSI values for both brown bullhead and walleye varied significantly among the sampling sites. Walleye from the Menominee River had a significantly higher HSI than those from the Fox River (Table 1); HSI in walleye did not differ between sexes. Two-way analysis of variance on brown bullhead indicated both sex and site differences, the HSI of females (0.027) being significantly higher than that of males (0.002). Consequently, we made site comparisons by sex. The HSIs of brown bullhead from the Cuyahoga River and Munuscong Lake were similar, as were those of brown bullhead from the Fox and Menominee rivers. The HSI of brown bullhead from the Cuyahoga River was significantly higher than that of brown bullhead from Fox and Menominee rivers, and the HSIs of brown bullhead were significantly greater in Munuscong Lake than in the Menominee River.

TABLE 1. Mean HSI (liver weight/body weight) for fish collected at tumor survey sites. Standard errors are in parentheses; values followed by different letters are significantly different ($p < 0.05$).

Site	Brown bullhead	No. of fish	Walleye	No. of fish
Fox River	0.0220 ab (0.0012)	31	0.0102 d (0.0006)	37
Menominee River	0.0202 a (0.0012)	39	0.0133 e (0.0005)	40
Munuscong Lake	0.0255 bc (0.0007)	68	0.0120 de (0.0006)	56
Cuyahoga River	0.0257 c (0.0007)	71	NA	

TABLE 2. Numbers of bullhead captured (*N*), the percent examined by gross observation (GO) and by random histopathology (RH), and the percent incidence of liver neoplasms (NEO) and potentially preneoplastic lesions (hepatocellular alteration or cholangiofibrosis) (PN) by age class for four Great Lakes tributaries.

	Age							
Site	2	3	4	5	6	7	8	9
Fox[a]								
N	8	16	13	4	1			
%GO	100	94	68	80	100			
%RH	0	6	32	20	0			
%NEO	0	0	0	0	0			
%PN	0	0	0	0	0			
Menominee[a]								
N	10	17	11	9				
%GO	100	88	64	44				
%RH	0	12	36	66				
%NEO	0	0	0	0				
%PN	0	0	0	0				
Munuscong								
N	2	7	6	15	17	8	6	2
%GO	50	86	100	87	76	75	67	100
%RH	50	14	0	13	24	25	33	0
%NEO	0	0	0	6.7	5.9	12.5	16.7	0
%PN	0	28.6	16.7	0	5.9	0	0	0
Cuyahoga								
N	16	33	26	5	4	1		
%GO	94	91	77	80	75	100		
%RH	6	9	23	20	25	0		
%NEO	0	6.1	7.7	60	25	0		
%PN	0	12.1	19.2	20	0	0		

[a]Numbers represent brown and black bullhead combined.

Histopathology

In general, walleye had a lower incidence of neoplasms than did brown bullhead in waters where both were collected. Only one hepatocellular neoplasm was found in 40 walleye examined from the Fox River, although areas of hepatocellular alteration were seen in two walleye from the Menominee River ($N = 56$) and one walleye from Munuscong Lake ($N = 40$). Three walleye from Munuscong Lake also had dermal ossifying fibromas and one had a hemangioendothelioma.

External neoplasms were seen only in both black and brown bullhead from the Fox river (7.7%; $N = 52$) and in brown bullhead from the Cuyahoga river (8.9%; $N = 90$). The majority of these lesions were squamous carcinomas in brown bullhead from the Cuyahoga (75%) river whereas 75% of external neoplasms in the Fox River brown bullhead were epidermal papillomas.

Liver neoplasms occurred in brown bullhead from Munuscong Lake and the Cuyahoga River (Table 2), as well as in brown bullhead sampled in 1983 from the Black River (Baumann et al. 1990). Similarly, areas of hepatocellular alteration and cholangiofibrosis also occured only in the livers of brown bullhead from Munuscong Lake and the Cuyahoga River (Table 2). In both rivers the peak incidence of these potentially preneoplastic lesions occurred at an earlier age than the peak incidence of neoplasms. When only randomly selected fish subjected to histopathology are considered, about one third of the Munuscong Lake brown bullhead and almost two thirds of those from the Cuyahoga River had either neoplastic or prenoeplastic liver lesions. At all locations, the frequency of grossly observable tumors was lower than that found by histopathology. Brown bullhead at age 4 from the Cuyahoga River were found to have liver cancer, while no liver cancer was found in Munuscong brown bullhead younger than age 6. However, only 11 brown bullhead from the Cuyahoga River and 12 from Munuscong Lake were subjected to random histopathology.

Chemical Contaminants

Four of five elements analyzed were highest in Cuyahoga River sediment (Table 3). Cadmium was also highest in brown bullhead from the Cuyahoga (Table 4). However, brown bullhead from Munuscong Lake had the highest concentrations of Cr and Ni, although these values were influenced by outliers which may have been contaminated. Munuscong Lake had the lowest sediment concentrations for all elements except Cr, which was lower in the Menominee River. Arsenic concentrations in sediment were 8–40 times higher in the Menominee River than at the other locations. Similarly, brown bullhead from the Menominee had 3–5 times more As in their tissues than did brown bullhead from the other sites.

Carcinogenic PAH were highest in the Black River, being about 200 times more concentrated than in Munuscong Lake sediment, where PAH were lowest (Table 3). However, a known point source in the Black River enabled us to sample the most contaminated sediments whereas the sediments in Munuscong and at the other sampling sites were composites of samples taken from several locations. Cuyahoga River sediment contained the next highest concentrations of PAH, the carcinogens being 3–60 times higher than in sediments from the other sites. Similarly, totals of selected noncarcinogenic PAH were approximately 10 times higher in brown bullhead from the Cuyahoga River than in brown bullhead from the other locations sampled (Table 4).

Polychlorinated aromatic compounds were highest in Fox River sediment, occurring at 8–400 times the concentrations at the other sites for individual groups of compounds (Table 3). Munuscong Lake sediment was lowest in polychlorinated dibenzo-*p*-dioxin (PCDD) and Black River sediment was lowest in polychlorinated dibenzofuran (PCDF) and non-ortho PCB. Brown bullhead from both the Fox and Menominee rivers had higher concentrations of polychlorinated aromatics than did those from the Cuyahoga; fish from Munuscong Lake had low

TABLE 3. Concentrations of selected contaminants in sediment from five Great Lakes tributaries. ND = not detected.

Contaminant	Menominee River	Fox River	Munuscong Laske	Cuyahoga River	Black River
		Inorganics (μg/g dry sediment)			
Arsenic	175[a]	9.2	4.0	21.2	14.2
Beryllium	<2	<2.0[a]	<2.0	2.0[a]	<2.0
Cadmium	<3	3.0[a]	<3.0	16[a]	10
Chromium	34	66[a]	57	111[a]	85
Nickel	24	34[a]	22	70[a]	60
Total	238	114.20	88	229.20	171.20
		Polynuclear aromatic hydrocarbons (μg/g dry sediment)			
Benz(*a*)anthracene	0.94	0.70	0.046	2.20	11.00
Benzo(*b*), (*j*), (*k*) fluoranthenes	1.20	1.90	0.091	4.60	15.00
Benzo(*a*)pyrene	0.66	1.00	0.044	2.60	8.80
Indeno(1,2,3-*c*,*d*) pyrene	0.18	ND	ND	1.40	6.40
Total	2.98	3.60	0.181	10.80	41.20
		Polychlorinated aromatic compounds (ng/g dry sediment)			
PCDDs[b] (Cl_4–Cl_7)	0.04	8.90	0.02	0.42	1.25
PCDFs[c] (Cl_4–Cl_7)	0.43	8.40	0.02	0.37	0.01
Non-ortho PCBs[d] (Cl_4–Cl_6)	0.14	1.40	0.05	0.10	ND[b]
Total	0.97	18.70	0.09	0.89	1.26

[a]Values given are the mean of two determinations.
[b]Polychlorinated dibenzo-*p*-dioxins.
[c]Polychlorinated dibenzofurans.
[d]Polychlorinated biphenyls.

levels of these compounds (Table 4). PCDD and PCDF with eight chlorines were not included in these totals, since these compounds are comparatively abundant and do not appear to be as biologically active.

Discussion

Liver tumors were found in both walleye and brown bullhead collected from Great Lakes tributaries and embayments. Although lesions were fewer and less advanced in walleye than in brown bullhead in Munuscong Lake, walleye were the only species from the Fox and Menominee in which liver lesions were detected. Even though the frequency of these lesions was low, all were documented from a small number of fish (10 per location) taken at random for histopathology. A larger study would be required to determine a frequency of neoplasms or cellular alteration with confidence. The lack of any lesions in the brown bullhead from these locations subjected to histopathology, including the lack of preneoplastic lesions, helps to confirm the lack of liver neoplasms observed by gross examination in that species, although again the sample size was small.

The HSIs for brown bullhead had the same rank order for locations as did neoplasm frequencies, the HSIs being significantly greater in the Cuyahoga River than either the Fox or Menominee. The HSI was also determined for brown bullheads sampled in 1981 from the Black River and from Buckeye Lake, a reference site (Fabacher and Baumann 1985). Samples of 10 or more fish from the Buckeye Lake – Black River study yielded HSIs of 0.0348 ± 0.0027 (mean ± SE) and 0.0340 ± 0.0018 for males and females, respectively, from the Black River and 0.0201 ± 0.0022 for males from Buckeye Lake. The Buckeye Lake HSI was almost identical to the lowest brown bullhead HSI in the 1984 survey sites (Menominee River) whereas the 1981 Black River HSI was 30% greater than that for Cuyahoga. Buckeye Lake brown bullhead and sediments contained low concentrations of PAH (Baumann and Harshbarger 1985), and no liver tumors were found in the fish (Baumann et al. 1987)

Other studies have also indicated a relationship between HSI and levels of contamination. The HSIs were significantly higher in bream (*Abramis brama*) collected from the Rhine River and its tributaries than in bream from less polluted locations (Sloof et al. 1983). Similarly, HSIs were higher in insecticide-resistant mosquitofish (*Gambusia affinis*) living in pesticide-contaminated drainage ditches than in those at an uncontaminated location (Chambers and Yarbrough 1982). Enlarged livers could result in increased activity of hepatic mixed-function oxidase (MFO) enzymes and thus an increased ability to metabolize xenobiotics (Fabacher and Baumann 1985; Chambers and Yarbrough 1982). Elevated MFO levels in fish were associated with PAH contamination at three Newfoundland locations (Payne and Penrose 1975; Payne 1976) and with oil spills in the Mediterranean Sea (Kurelec et al. 1977) and in Massachusetts (Burns 1976; Stegeman 1978).

The HSIs were low in both brown bullhead and walleye from the Fox River, even though organochlorines were highest at this location. Also, HSIs for both species were somewhat elevated at Munuscong Lake, where levels of carcinogens in sediment samples were low. Although HSIs can vary with

TABLE 4. Average concentrations of selected contaminants in brown bullhead from four Great Lakes tributaries. Standard deviations are given in parentheses; ND = not detected.

Contaminant	Fox River	Menominee River	Munuscong Lake	Cuyahoga River
		Inorganics (ng/g wet weight)		
Arsenic	30 (20)	160 (50)	40 (30)	50 (20)
Beryllium	ND	ND	ND	ND
Cadmium	5 (2)	10 (6)	30 (16)	130 (10)
Chromium	70 (20)	120 (60)	570 (960)[a]	80 (50)
Nickel	260 (160)	820 (930)	8100 (17 400)[a]	290 (100)
Total	355	1110	8740	550
		Polynuclear aromatic hydrocarbons (ng/g wet weight)		
Phenanthrene	6.8 (7.86)	8.2 (4.18)	5.2 (10.031)	75.2 (53.327)
Anthracene	0.7 (1.49)	0.2 (0.632)	0.4 (1.26)	31.4 (20.430)
Fluoranthene	9.8 (7.96)	15.6 (14.30)	17.5 (18.332)	65.5 (31.085)
Pyrene	0.6 (1.90)	0	1.9 (3.725)	39.6 (12.799)
Chrysene	2.0(4.447)	0	0	8.5 (7.835)
Total	20	24	24	220
		Polychlorinated aromatic compounds (pg/g wet weight)		
PCDDs[b] (Cl_4–Cl_7)	21	6	4	23
PCDFs[c] (Cl_4–Cl_7)	13	13	2	32
Non-ortho PCBs[d] (Cl_4–Cl_6)	272	470	30	48
Total	306	489	36	103

[a]High averages and large standard deviations for Cr and Ni were caused by two fish with outlier concentrations for these elements. The possibility exists that these samples were contaminated during homogenization or contained large amounts of ingested sediment.

[b]Polychlorinated dibenzo-*p*-dioxins.

[c]Polychlorinated dibenzofurans.

[d]Polychlorinated biphenyls.

nutrition, disease, and season (Sloof et al. 1983; Chambers and Yarbrough 1982), fish condition and disease incidence did not vary with HSIs in our samples, and the index might be useful as an indicator of carcinogenic PAH exposure in brown bullhead. At least in that species, HSI does not appear to be greatly affected by environmental exposure to organochlorines or As.

No liver neoplasms were found in brown bullhead from the Fox or the Menominee, even though sediments from the Fox were highest in PCDD, PCDF, and non-ortho PCB, and brown bullhead from the Fox and Menominee rivers had highest total concentrations of these same compounds. Similarly, both sediments and fish from the Menominee were highest in As. Although this does not rule out all possibility of carcinogenic effects on brown bullhead by these compounds, it indicates that these organochlorines and As are not a primary cause of neoplasm epizootics in this species.

The frequency of liver neoplasms in English sole from Puget Sound was not statistically correlated with organochlorine concentrations in sediment (Malins et al. 1984). Furthermore, laboratory studies verified that simultaneous exposure to PCB altered benzo(*a*)pyrene (BaP) metabolism, resulting in a higher proportion of glutathione-*S*-transferase conjugates (Varanasi et al. 1987). Notably, exposure to PCB resulted in lower frequencies of PAH-induced neoplasms in rodents (DiGiovanni and Slaga 1981), and simultaneous exposure of rainbow trout (*Oncorhynchus mykiss*) to PCB reduced the incidence of liver cancer induced by aflatoxin B_1 (Hendricks et al. 1977). Thus, high concentration of organochlorines in the Fox River might have inhibited PAH-induced carcinogenesis.

Fabacher et al. (1988) found that the A_2 and A_3 fractions of crude organic extracts from Munuscong Lake were marginally mutagenic in the Ames bacteria assay and induced DNA repair in rat hepatocytes, indicating that substances in the sample had mutagenic activity in metabolically competent cells as well. In the Chinese hamster ovary hypoxanthine-quanine phosphoribosyl transferase assay the dose–response slope was marginally significant ($p < 0.05$) for the Lake Munuscong A_2 fraction, even though the responses were not significantly different from those of controls. These observations were consistent with the presence of low levels of BaP and other known petroleum-derived mutagens in the A_2 fraction from this site.

Even though the sediment extracts from Munuscong Lake were slightly mutagenic, levels of all potential carcinogens analyzed in sediment were lower at Munuscong Lake than at the other locations. Nevertheless, liver neoplasms were found in fish from that location. Possible explanations include a carcinogenic compound that we did not analyze and identify, sediment sampling that missed the exposure site (either geographically or temporally), and a viral etiology for the neoplasms. However, no publication demonstrating a viral etiology for liver neoplasms in fish is known to us, and no specimens of liver neoplasms are listed as having a viral etiology in the collection of the Registry of Tumors in Lower Animals of the Smithsonian Institution.

The incidence of neoplasms in Munuscong Lake brown bullhead also differed by being distributed over a greater range of age classes. Our sample of the Munuscong population even had an older age class distribution than did that of Muskellung Lake, New York (Sinnott and Ringler 1987), and Old Woman Creek, Ohio (Baumann et al. 1990), two relatively unpolluted locations. The oldest brown bullhead captured at these two sites were age 7 whereas brown bullhead of ages 8 and 9 were taken at Munuscong. Neoplasms were first found in Munuscong Lake brown bullhead at age 5 and in Cuyahoga River brown bullhead at age 3. Similarly, cancer occurred in Cuyahoga River fish at age 4, but was not found in Munuscong Lake fish until age 6. The frequency of liver cancer ($N = 48$) was 31% by age 3 in brown bullhead from the Black River in 1982 (Baumann et al. 1990). Although the sample size is small, these differences suggest a faster progression of neoplasms in the Black and Cuyahoga. Such accelerated tumor development could be caused by exposure to a more potent or more concentrated carcinogen (Aoki and Matsudaira 1984), exposure to a fixed dosage of carcinogen for a longer time (Aoki and Matsudaira 1984), or exposure and development occurring at higher water temperatures (Kyono-Hamaguchi 1984).

Liver tumor frequencies in brown bullhead were highest in the Cuyahoga River, where PAH levels were highest in fish tissue. Both the Cuyahoga and the Black rivers also had higher concentrations of PAH in the sediment than did other locations. Although brown bullhead were not sampled from the Black during this study, 125 collected in 1982 had a 60% liver neoplasm frequency (Baumann et al. 1990). Sediments from both the Cuyahoga and the Black were also found to be mutagenic in both unscheduled DNA synthesis with rat hepatocytes and in the Chinese hamster ovary hypoxanthine-quanine phosphoribosyl transferase assay (Fabacher et al. 1988). PAH have previously been implicated as a cause of liver neoplasms in brown bullhead from the Black (Baumann et al. 1987). Neoplasms have also been associated with PAH in English sole from several locations in Puget Sound (Malins et al. 1984) and in several freshwater species from eastern Lake Erie and the Niagara River (Black 1983). Additional evidence from various studies linking neoplasms in fish to PAH was summarized in a recent review article (Baumann 1989).

Elevated levels of DNA-aromatic adducts, believed to indicate the initiation phase of carcinogenesis, have been found in brown bullhead taken from the Buffalo and Detroit rivers (Dunn et al. 1987; Maccubbin et al. 1990) and from the Cuyahoga (Stalling et al. 1991). Brown bullhead are able to metabolize BaP to carcinogenic metabolites which then bind to DNA (U. Varanasi, National Marine Fisheries Service, 2724 Montlake Blvd. E., Seattle, WA, pers. comm.). The brown bullhead may be one of the more susceptible species to PAH-induced neoplasms, since the fish metabolizes a greater percentage of BaP to carcinogenic metabolites than do rats (Swain and Melius 1984). Cultured cells from brown bullhead also conjugated PAH metabolites to glutathione and glucuronic acids less rapidly than cells from bluegill (*Lepomis macrochirus*) or rainbow trout (Smolarek et al. 1987), and rainbow trout fed dietary BaP had a 15% incidence of liver neoplasms by 12 mo (Hendricks et al. 1985).

Our findings are consistent with the growing body of evidence linking liver neoplasms in wild populations of brown bullhead with elevated concentrations of PAH in sediment. Brown bullhead from the location having the highest PAH sediment concentrations had the highest frequency of liver neoplasms. We have also identified a brown bullhead population with an elevated liver tumor frequency that is seemingly associated with relatively unpolluted sediment. This indicates either the problem of using sediment concentrations or current tissue residue levels as a substitute for exposure values, or the lack of knowledge about all possible causes of brown bullhead liver neoplasia, or both. In retrospect, our study would have been more informative if we had examined the livers of all fish by histopathology. Gross examination is insufficient to identify any but the most advanced lesions, thus making estimates of tumor frequencies conservative. Populations having tumor frequencies that are relatively low, yet elevated over background levels, will be identified only by large-scale studies in which all livers are subjected to histopathology.

Acknowledgements

We thank L. M. Smith for carrying out the PCDD and PCDF analyses, W. Brumbaugh and T. May for performing analyses of elements, and T. A. Jodon, E. P. Johnston, and V. A. Murchake for helping us collect and process the fish. Kathy Price and Jennifer Clark provided the histopathology. Phyllis Spero assembled the pathology data and typed the pathology reports.

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Metabolic Effects Associated with Chronically Elevated Cortisol in Rainbow Trout (*Oncorhynchus mykiss*)

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Andersen, D. E., S. D. Reid, T. W. Moon, and S. F. Perry. 1991. Metabolic effects associated with chronically elevated cortisol in rainbow trout (*Oncorhynchus mykiss*). Can. J. Fish. Aquat. Sci. 48: 1811–1817.

Carbohydrate and protein metabolism were examined in rainbow trout, *Oncorhynchus mykiss*, fitted with mini-osmotic pumps which maintained plasma cortisol levels at approximately 100 or 200 ng·mL^{-1} for 10 d. Plasma glucose, lactate, and protein levels were unaffected by 10 d of cortisol administration, despite a significant elevation in plasma cortisol. Plasma amino acids in cortisol-treated fish (1023.8 ± 90.7 μg·mL^{-1}) were significantly elevated compared with shams (716.7 ± 68.5 μg·mL^{-1}) after 9 d. Liver glycogen content was significantly reduced by cortisol treatment. The activities of the liver enzymes assayed were unchanged; likewise the flux of radioactive substrates to radiolabeled CO_2, glucose, and protein in isolated hepatocytes was unaffected in trout with chronically elevated cortisol compared with shams. The glucose replacement rate (Ra) was unchanged after 2 wk of cortisol treatment. These data do not support the purported role of cortisol as a glucocorticoid in rainbow trout. While chronically elevated cortisol may increase the supply of plasma amino acids, the hormone does not appear to alter the manner in which this potential gluconeogenic substrate is metabolized. The absence of other stressors may be partially responsible for the differences between this study and others in the literature.

On a étudié le métabolisme des glucides et des protéines chez des truites arc-en-ciel, *Oncorhynchus mykiss*, qui ont porté pendant 10 jours une mini-pompe osmotique maintenant le cortisol plasmatique à environ 100 ou 200 ng·mL^{-1}. Les teneurs plasmatiques en glucose, lactate et protéines étaient inchangées au bout de dix jours de traitement au cortisol, malgré une augmentation sensible du cortisol plasmatique. Chez les poissons traités, les concentrations d'acides aminés plasmatiques (1 023,8 ± 90,7 μg·mL^{-1}) étaient supérieures dans une mesure significative à celles mesurées chez les témoins (716,7 ± 68,5 μg·mL^{-1}) au bout de 9 jours. Le glycogène hépatique a sensiblement baissé avec le traitement au cortisol. L'activité des enzymes hépatiques évaluées est restée inchangée; de même, le flux de substrats radioactifs vers les CO_2, glucose et protéines radiomarqués dans des hépatocytes isolés n'a pas été modifié chez la truite à cortisol chroniquement élevé comparé aux témoins. Le taux de renouvellement du glucose (Ra) était inchangé après deux semaines de traitement au cortisol. Ces résultats ne confirment pas l'hypothèse selon laquelle le cortisol joue le rôle d'un glucocorticoïde chez la truite arc-en-ciel. L'élévation chronique du cortisol peut faire augmenter l'apport d'acides aminés plasmatiques, mais il semble que le cortisol n'influe pas sur la façon dont ce substrat gluconéogène potentiel est métabolisé. L'absence d'autres facteurs de perturbation explique peut-être en partie les différences qu'on note entre cette étude et celles dont on fait état dans la littérature du domaine.

Received September 26, 1990
Accepted April 4, 1991
(JA741)

Reçu le 26 septembre 1990
Accepté le 4 avril 1991

Cortisol has a variety of metabolic effects in mammals (for review see Kraus-Friedmann 1984). It is considered to be a glucocorticoid, increasing plasma glucose concentration by the stimulation of both gluconeogenesis and glycogenolysis in hepatic tissue. The catabolic effect of cortisol on muscle protein is well documented and results in readily available gluconeogenic substrates in the form of circulating amino acids.

The metabolic effects of cortisol in teleost fish remain controversial. Chan and Woo (1978) demonstrated that in the Japanese eel, *Anguilla japonica*, a single intramuscular (IM) cortisol injection (1 mg·kg^{-1}) caused a significant elevation in plasma glucose and amino acid levels. This protocol also stimulated liver glycogen deposition and increased the activity of the gluconeogenic enzyme, glutamic-oxaloacetic transaminase (GOT). Conversely, the activities of glutamic-pyruvic transaminase (GPT), fructose-1,6-diphosphatase (FDPase), and glucose-6-phosphatase (G6Pase) were unaffected. In *Fundulus heteroclitus*, daily cortisol injections (20 mg·kg^{-1}) for 5 d resulted in hyperglycaemia, and in starved fish an increase in liver glycogen concentrations (Leach and Taylor 1982). Neither protein nor amino acid metabolism was affected in that study. Lidman et al. (1979) produced similar results in European eels, *A. anguilla*, using a slightly modified protocol (5 mg·kg^{-1}) for 10 d. More recently, Foster and Moon (1986) showed that in the American eel, *A. rostrata*, cortisol treatment (0.35 mg·kg^{-1}·d^{-1}) for 10 d produced hypoglycaemia. Unexpectedly, this study also reported a decline in the overall flux of ^{14}C-labeled substrates to [^{14}C] glucose in isolated hepatocytes, implying a decreased gluconeogenic activity, while activities of liver phosphoenolpyruvate carboxykinase (PEPCK) were elevated, suggesting an increased gluconeogenic activity. Daily intraperitoneal (IP) injections of cortisol (20 mg·kg^{-1}) for 5 d in rainbow trout, *Oncorhynchus mykiss*, increased the activities of the transaminase enzymes, GOT and GPT, implying an

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increase in the potential of amino acids as gluconeogenic substrates (Freeman and Idler 1973). Interestingly, that study also reported that five daily injections of metopirone (50 mg·kg^{-1}) (Ciba Ltd.) increased GOT activity, yet this drug is known to impair glucocorticoid synthesis in rainbow trout by inhibiting anterior pituitary function (Fagerlund et al. 1968).

Given the discrepancies in the literature, there clearly is a requirement for further research on the metabolic effects of cortisol in teleost fish. The mode of cortisol administration may be confounding the results of some of these previous studies. If the metabolic effects of cortisol are to be isolated from those of other stress-induced changes (i.e. an elevation in circulating catecholamines), then it is essential that the hormone be administered in such a way as to minimize the disturbance to the animal. The majority of studies that have examined the metabolic effects of cortisol, however, have relied on frequent IP injections to chronically elevate plasma cortisol levels. Foster and Moon (1986) demonstrated that this technique caused a massive postinjection elevation of plasma cortisol levels followed by its rapid clearance. After 3 d of treatment, cortisol levels in treated fish were significantly below control levels. Owing to these large and continual oscillations in plasma cortisol content, this mode of administration probably does not reflect the glucocorticoid status of teleost fish subjected to chronic stress. A recent study by Vijayan and Leatherland (1989) used IP implants of hydrogenated coconut oil containing cortisol to address this administration problem.

This study examines the effect of chronically (≥ 10 d) elevated plasma cortisol levels on carbohydrate and protein metabolism in rainbow trout. Mini-osmotic pumps (Alza Corp., Palo Alto, CA) were used to administer cortisol at a constant dosage, thereby eliminating the stress associated with netting and daily injections and the massive oscillations in the cortisol levels accompanying daily injection protocols. Plasma cortisol levels were measured daily and the concentrations of plasma glucose, amino acids, and lactate were measured throughout the study. The activities of selected hepatic enzymes and the metabolic flux of two gluconeogenic precursors (alanine and lactate) were measured using isolated hepatocytes to establish the role of cortisol in the regulation of hepatic gluconeogenesis. Finally, the potential effects of cortisol on glucose turnover were assessed.

Materials and Methods

Rainbow trout were purchased from either Thistle Springs Trout Farm (Ashton, Ont.) or Linwood Acres Trout Farm (Campbellcroft, Ont.). Fish were held in 500-L fibreglass tanks supplied with flowing, dechlorinated, and well-aerated City of Ottawa tap water. The chemical composition of this water has been previously reported (Perry and Laurent 1989). Photoperiod (12L:12D) and temperature (13 ± 1°C) were held constant throughout the experiment. Fish were fed daily to satiation with commercial trout pellets (Purina Trout Chow) prior to the experiments. Fish were permitted a minimum of 2 wk to acclimate to laboratory conditions prior to all experiments. All experiments were performed between May and August. The fish used in experiments I, II, and III had a mean weight of 178.1 ± 6.7 g. In experiment IV the mean weight of the fish was 279.1 ± 16.5 g. In all cases, fish were sexually immature; thus, size would not be expected to affect the manner in which they respond to cortisol treatment.

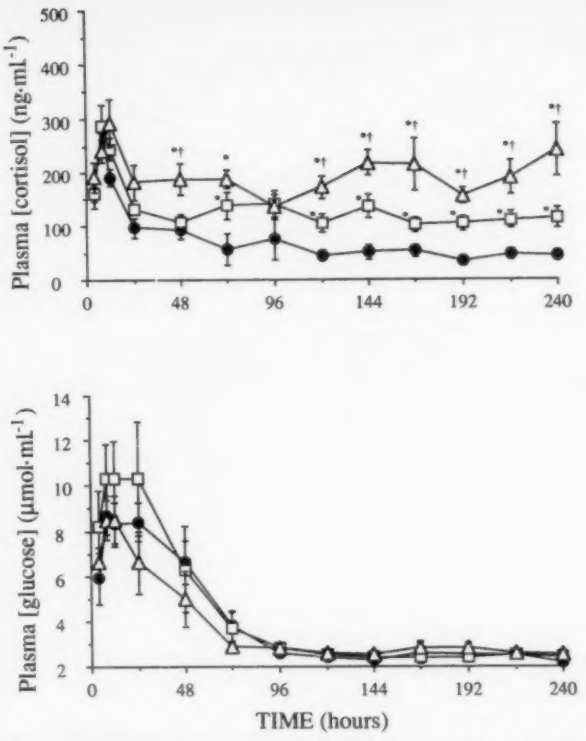

FIG. 1. Time course of plasma cortisol and glucose in sham and cortisol-treated fed rainbow trout. Solid circles, shams; open squares, cortisol administered at 4.2 μg·h^{-1}·100 g^{-1}; open triangles, cortisol administered at 8.4 μg·h^{-1}·100 g^{-1}. Vertical bars through each point represent 1 SEM. *Significantly different sham-treated fish; † significantly different from fish with cortisol elevated to 100 ng·mL^{-1}.

Experiment I. Effect of Cortisol on Plasma Metabolites

Fish were anaesthetized in a 1 : 10 000 (w/v) solution of 3-aminobenzoic acid ethyl ester (MS-222, Sigma Chemical Co.) adjusted to pH 7.0–7.5 with sodium bicarbonate. Indwelling dorsal aortic cannulae (Soivio et al. 1975) were surgically fitted and a mini-osmotic pump (model 2001; 200 μL total volume) placed in the peritoneal cavity through a small incision, which was sutured closed after implantation. After surgery, fish were placed in black plexiglas boxes (volume 3 L) supplied with identical water as above. Food was not offered, as fish will not feed under these conditions. Two experimental groups of fish were used for this experiment. Group 1 received osmotic pumps which delivered cortisol, dissolved in 50% Molecusol HBP (Pharmatec, Inc. Alachua, FL), at a continuous rate of 4.2 μg·h^{-1}·100 g^{-1}. This pumping rate maintained plasma cortisol levels near 100 ng·mL^{-1}. Group 2 fish received pumps which administered cortisol at a rate of 8.4 μg·h^{-1}·100 g^{-1} resulting in plasma cortisol levels near 200 ng·mL^{-1}. Sham fish were fitted with pumps which delivered only the Molecusol HPB vehicle. Operations were performed in the morning (08:00–12:00) and a 500-μL blood sample was taken 4, 8, and 12 h following surgery through the dorsal aortic cannula. Thereafter, a 500-μL blood sample was taken at 11:00 each day for the following 10 d. Blood was centrifuged for 15 s at 12 000 × *g* (Fisher Micro-centrifuge model 235B) immediately after collection. The plasma was removed, frozen in liquid nitrogen,

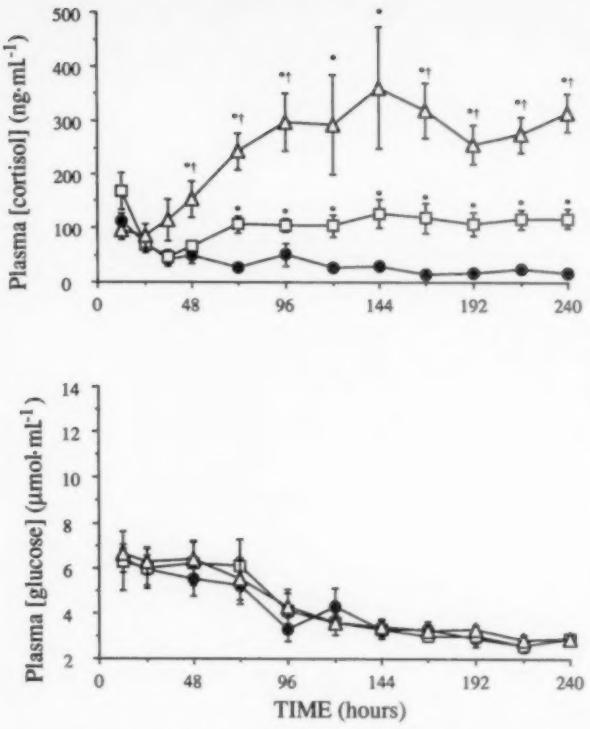

FIG. 2. Details as in Fig. 1, except fish were deprived of food for 3 wk prior to experiment.

and stored at −70°C. Red blood cells were resuspended in heparinized (10 units·mL^{-1}) Cortland saline (Wolf 1963) and reinjected into the animal through the cannula. Plasma cortisol concentrations were measured in all samples using a radioimmunoassay kit (ICN Biomedicals, Carson, CA); the protein content of standards was adjusted to reflect values found in fish plasma, which increased assay sensitivity at low cortisol concentrations. Plasma glucose was measured enzymatically according to Bergmeyer (1983).

Insulin may counteract the effects of elevated cortisol on plasma glucose (Rizza et al. 1981). Therefore, a subsequent set of experiments was performed using fish which were deprived of food for 3 wk prior to mini-osmotic pump implantation. This period of starvation has been shown to significantly depress insulin levels in trout (Moon et al. 1989). The experimental protocol was identical to that described above, except that the first blood sample was withdrawn 12 h after surgery. Plasma ninhydrin positive substances (amino acids) by the method of Troll and Cannan (1953) and plasma osmolarity were measured using a vapour pressure osmometer (Wescor, Inc.).

Experiment II. Effect of Cortisol on Liver Enzyme Activities

Mini-osmotic pumps were implanted using the same protocol as above. The pumps delivered cortisol at a rate of 4.2 μg·h^{-1}·100 g^{-1} resulting in plasma cortisol levels near 100 ng·mL^{-1}. Sham fish were fitted with pumps which delivered only the Molecusol HPB vehicle. The fish were returned to a 500-L tank after surgery and resumed feeding 24 h later. Fish were fed to satiation once daily for 8 d following surgery. Ten days after pump implantation, a blood sample (2.0 mL) was withdrawn from the caudal vein/artery using a syringe rinsed with heparin (2500 units·mL^{-1}). Plasma was recovered and stored as above. Fish were killed by a sharp blow to the head and the livers were immediately excised and frozen between aluminum blocks cooled in liquid nitrogen. Livers were stored at −70°C until analyzed.

Plasma cortisol and glucose were analyzed as in experiment I. Plasma lactate was measured enzymatically according to Bergmeyer (1983) and plasma protein using Folin's reagent (Lowry et al. 1951).

Liver enzyme activities were determined spectrophotometrically (Beckman DU-65, Fullerton, CA) at 10°C. Frozen liver pieces were homogenized with a Polytron PCU-2 (Brinkmann Instruments Co., Toronto, Ont.) in 4 volumes of a phosphorylation–dephosphorylation "stopping buffer." All enzyme assays were performed as in Moon et al. (1989) and presented as activity units (micromoles per minute) per gram of liver wet weight. Unless noted, all activities reported are optimal under the conditions established.

Experiment III. Effect of Cortisol on the Flux of ^{14}C-Labeled Substrates in Isolated Hepatocytes

Experimental and sham fish were treated as in experiment II. Isolated hepatocytes were prepared 10 d after pump implantation by the collagenase perfusion method of Foster and Moon (1989). Isolated cells in resuspension medium (1 mL) were added to 20-mL glass vials containing either 5 mmol alanine ·L^{-1} or 5 mmol lactate ·L^{-1}, and 150 000 dpm of the equivalent ^{14}C-labeled substrate (NEN Canada) was added to the appropriate vial (specific activities: alanine, 2627 MBq·mmol^{-1}; lactate, 2638.1 MBq·mmol^{-1}). Cells were incubated at 18°C for 2 h prior to termination by the addition of 100 μL of 70% perchloric acid. The flux of radioactive substrates to radiolabeled CO_2, glucose, and protein was determined as in Foster and Moon (1986); production rates were determined from initial substrate specific activity values.

Experiment IV. Effect of Cortisol on Glucose Turnover

Trout were fitted with mini-osmotic pumps and returned to 500-L fibreglass tanks as in experiment II. Fourteen days later, fish were fitted with indwelling dorsal aortic cannulae which permitted periodic blood sampling (as in experiment I). Immediately following surgery, fish were placed into the individual black boxes and were allowed to recover for 3 d, as in experiment I. Glucose turnover experiments were undertaken using the protocol of Bever et al. (1977).

Fish were injected with 370 kBq of [6−^{3}H]glucose (NEN Canada) in 250 μL of saline followed immediately by 250 μL of heparinized (10 units·mL^{-1}) saline to flush the cannula. Blood was sampled at 1, 2, 3, 4, 6, 8, 12, 16, 22, and 28 h after dosing. Blood samples were centrifuged and the plasma removed and stored at −70°C until analyzed; red cells were reinjected as noted previously. Plasma cortisol was determined as previously described. Labeled plasma glucose was extracted using a two-column resin extraction system (Dowex-1 1X8, 50–100 mesh followed by Dowex 50X8, 100–200 mesh) to eliminate other possible labeled intermediates (Foster and Moon 1986). This extracted glucose was counted using a liquid scintillation counter (LKB Rackbeta 1211, Pharmacia) and glucose specific activity determined. Plasma glucose was assayed as above. Glucose replacement rate (Ra) was calculated according to Katz et al. (1974). Briefly, the injected dose was divided by

TABLE 1. Plasma osmolarity and total amino acid concentrations from unfed (3 wk) rainbow trout fitted with mini-osmotic pumps which delivered either cortisol or vehicle (sham). Blood was sampled through a dorsal aortic catheter 24, 96, and 216 h after the pumps were implanted. Rates of cortisol administration were either 4.2 $\mu g \cdot h^{-1} \cdot 100\ g^{-1}$, resulting in plasma cortisol concentrations near 100 $ng \cdot mL^{-1}$ (group 1), or 8.4 $\mu g \cdot h^{-1} \cdot 100\ g^{-1}$ (group 2), resulting in plasma cortisol concentrations near 200 $ng \cdot mL^{-1}$.

Time since implant	Sham	Group 1 cortisol (100 $ng \cdot mL^{-1}$)	Group 2 cortisol (200 $ng \cdot mL^{-1}$)
Osmolarity (mosmol)			
24 h	293.2 ± 5.6	280.3 ± 2.5	295.3 ± 2.5
96 h	285.2 ± 3.0	284.3 ± 6.3	297.0 ± 4.1
216 h	288.8 ± 6.3	294.2 ± 3.4	303.7 ± 4.9
Amino acids ($\mu g \cdot mL^{-1}$)			
24 h	1078.8 ± 84.1	1044.5 ± 155.3	953.1 ± 88.8
96 h	961.0 ± 112.3	922.2 ± 103.9	1025.4 ± 111.3
216 h	716.7 ± 68.5[a]	1023.8 ± 90.7[b]	942.3 ± 63.7[b]

[a]Significantly different from 24-h sample, two-factor repeated measures ANOVA and Fisher's LSD, $p < 0.05$.

[b]Significantly different from sham treatment, two-factor repeated measured ANOVA and Fisher's LSD, $p < 0.05$.

TABLE 2. Liver enzyme activities ($\mu mol \cdot min^{-1} \cdot g$ wet wt^{-1}, except %GPase *a* and activity ratio) in sham and cortisol-treated fed rainbow trout. Cortisol was elevated to about 100 $ng \cdot mL^{-1}$ using mini-osmotic pumps. Fish were fed daily to satiation with commercial trout pellets 8 d after surgery. Values are mean activity ($units \cdot g^{-1}$) ± 1 SEM (*N*). Abbreviations: GPase, glycogen phosphorylase; PK, pyruvate kinase; PFK, phosphofructokinase; PEPCK, phosphoenolpyruvate carboxykinase; LDH, lactate dehydrogenase; GOT, glutamate-oxaloacetic transaminase; GPT, glutamate-pyruvic transaminase; GDH, glutamate dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase. Activity ratio = activity at low [PEP]/activity at high [PEP]; %GPase *a* represents percentage of total glycogen phosphorylase ($a+b$) in the active form (a).

Enzyme	Sham treatment	Cortisol treatment
GPase, total	18.5 ± 1.7 (7)	16.2 ± 1.7 (6)
%GPase *a*	73.0 (5)	70.0 (6)
PK, V_{opt}	70.5 ± 15.1 (8)	71.5 ± 14.7 (6)
Activity ratio	0.26 (8)	0.18 (6)
PFK	9.7 ± 2.2 (7)	10.1 ± 3.7 (6)
PEPCK	10.3 ± 1.8 (7)	8.1 ± 1.2 (5)
LDH	58.8 ± 8.7 (8)	58.9 ± 6.7 (6)
GOT	53.5 ± 5.5 (8)	48.1 ± 2.9 (6)
GPT	36.3 ± 6.3 (8)	34.1 ± 6.8 (5)
GDH	150.0 ± 24.3 (8)	171.0 ± 26.1 (6)
G6PDH	50.2 ± 3.8 (8)	65.6 ± 8.4 (6)

the area under the specific activity curve which was determined using a digitizer (Sigmascan, Jandel Scientific, Corte Madera, CA).

Statistical Analysis

Statistical analyses for experiment I were performed using two-factor repeated measures ANOVA with postanalysis comparison of means utilizing Fisher's least significant difference (LSD) test at $p < 0.05$. All other statistical analyses were performed using either paired or unpaired Student's *t*-tests at a level of significance of $p < 0.05$.

Results

Experiment I

A surge in plasma cortisol occurred immediately after surgery in both experimental and sham trout (Fig. 1). Plasma cortisol was significantly elevated in a dose-dependent manner within 72 h of pump implantation and remained elevated until fish were killed at 240 h. Mini-osmotic pumps, therefore, were an effective method for chronically elevating cortisol titers in trout.

TABLE 3. Plasma metabolites in rainbow trout 10 d after being fitted with mini-osmotic pumps which delivered either cortisol or vehicle (sham). Cortisol dosage rate was 4.2 $\mu g \cdot h^{-1} \cdot 100\ g^{-1}$. Values are means ± 1 SEM (*N*).

	Sham treatment	Cortisol treatment
Body weight (g)	173.6 ± 8.7 (9)	183.0 ± 10.7 (8)
Plasma cortisol ($ng \cdot mL^{-1}$)	32.0 ± 14.0 (9)	91.5 ± 21.1 (8)[a]
Plasma glucose ($\mu mol \cdot mL^{-1}$)	4.2 ± 0.4 (9)	3.7 ± 0.3 (8)
Plasma lactate ($\mu mol \cdot mL^{-1}$)	0.5 ± 0.07 (9)	0.6 ± 0.1 (8)
Plasma protein ($mg \cdot 100\ mL^{-1}$)	25.4 ± 0.8 (9)	26.1 ± 1.9 (8)

[a]Significantly different from control value, Student's *t*-test, $p < 0.05$.

A postsurgery elevation of plasma glucose occurred in both sham and cortisol-infused fish which lasted for 72 h (Fig. 1). There was, however, no dose-dependent response between glucose and cortisol concentrations at any time during the 240-h experimental period.

Repeating experiment I with unfed fish had little effect on either plasma cortisol or glucose levels (Fig. 2). The extent of the postoperative cortisol and glucose surges observed in the fed fish was, however, less apparent compared with the experiments done on the fed fish. The reason for these decreased postsurgery peaks is not understood, since one might expect a greater hyperglycaemia due to reduced insulin levels. It is possible that 3 wk of food deprivation reduces the normal stress response of a fish.

Plasma amino acid levels in sham fish decreased with time postsurgery, this decrease being significant by 216 h (Table 1). This trend towards decreasing plasma amino acids was not seen in cortisol-infused fish, where concentrations remained high throughout the experiment. By 216 h, levels were significantly higher than shams, but were not different from the 24-h samples. Plasma amino acid concentrations were unrelated to

TABLE 4. Rates of glucose and CO_2 production from radioactive alanine and lactate and substrate incorporation into protein by isolated rainbow trout hepatocytes. Hepatocytes were prepared from either sham or cortisol-treated (approximately 100 ng·mL plasma^{-1}) fish. Fish were fed and had implants for 10 d prior to sacrifice. Values are means ± 1 SEM (*N*).

	Sham treatment	Cortisol treatment
Initial glycogen (μmol·g^{-1})	42.6 ± 8.9 (8)	25.8 ± 5.6 (8)[a]
Lactate fluxes		
To protein (μmol·g^{-1}·h^{-1})	0.64 ± 0.13 (9)	0.61 ± 0.12 (8)
To CO_2 (μmol·g^{-1}·h^{-1})	1.62 ± 0.35 (9)	1.54 ± 0.22 (8)
Glucose production (μmol·g^{-1}·h^{-1})	0.56 ± 0.2 (9)	0.79 ± 0.3 (8)
Glycogen depletion (μmol·g^{-1}·h^{-1})	9.7 ± 2.6 (8)	6.1 ± 2.8 (8)
Total glucose production (μmol·g^{-1}·h^{-1})	3.0 ± 0.6 (8)	3.4 ± 0.8 (8)
Alanine fluxes		
To protein (μmol·g^{-1}·h^{-1})	0.59 ± 0.19 (9)	0.87 ± 0.14 (8)
To CO_2 (μmol·g^{-1}·h^{-1})	1.05 ± 0.21 (9)	1.67 ± 0.53 (8)
Glucose production (μmol·g^{-1}·h^{-1})	0.08 ± 0.03 (9)	0.10 ± 0.03 (7)
Glycogen depletion (μmol·g^{-1}·h^{-1})	8.6 ± 3.0 (8)	7.9 ± 3.6 (8)
Total glucose production (μmol·g^{-1}·h^{-1})	2.3 ± 0.5 (8)	2.9 ± 0.7 (8)

[a]Significantly different from control value, Student's *t*-test, $p < 0.05$.

cortisol content. Plasma osmolarity was not affected by cortisol treatment (Table 1).

Experiment II

Chronic cortisol elevation for 10 d had no significant effect on the optimal activity of a number of gluconeogenic and glycolytic enzymes (Table 2). The phosphorylation status of glycogen phosphorylase (GPase) and pyruvate kinase (PK) were also unaffected as indicated by no change in % GPase *a* and in the PK activity ratio. Furthermore, the plasma levels of glucose, lactate, and protein were unchanged by cortisol administration (Table 3).

Experiment III

The flux of radioactive lactate or alanine to protein, CO_2, and glucose in isolated hepatocytes was unaffected by the chronic administration of cortisol (Table 4). Initial hepatocyte glycogen concentration was significantly lowered by chronic cortisol administration, but neither glycogen depletion rate nor glucose production rate differed from shams.

It should be noted that the hepatocyte glycogen content of these particular fish was lower than in those in other studies undertaken in this laboratory (e.g. Foster and Moon 1989). To alleviate concerns about low initial glycogen levels, the experiment was subsequently repeated using fish from another supplier. These fish, however, had even lower hepatocyte glycogen contents. Except for differences in glycogen depletion and glucose production rates, no qualitative differences were noted between these experiments (data not presented). Such quantitative differences in glycogen depletion were expected based upon the differences in glycogen contents of the two groups of fish (Mommsen 1986; Foster and Moon 1990). The reason for hepatocyte glycogen levels below those previously reported remains unclear.

Experiment IV

Plasma glucose concentrations were unchanged in individual fish over the 28-h sampling time following a [6−^{3}H]glucose injection (Fig. 3, inset), even though differences existed between individuals. Such individual differences were unrelated to the treatment. The glucose kinetic curves (Fig. 3) followed a simple exponential function, and glucose replacement rates (Ra) (sham 0.020 ± 0.0049 (6) mg·min^{-1}·100 g^{-1}, cortisol 0.015 ± 0.0033 (6) mg·min^{-1}·100 g^{-1}) were not significantly different and were unrelated to cortisol treatment. These values are very close to those determined by Bever et al. (1977) in kelp bass (*Paralabrax* sp.). Glucose turnover, therefore, was unaffected by chronic cortisol administration. Glucose replacement and utilization rates were also measured using the equations from Bever et al. (1977). These calculations, which assume non-steady-state conditions, also indicated that glucose turnover was unaffected (data not shown).

Discussion

This study differs from previous investigations into the metabolic effects of cortisol in several ways. Mini-osmotic pumps were used to continuously deliver a prescribed cortisol dose to the circulation. Continuous infusion is preferable to daily IP injections used in many studies (e.g. Freeman and Idler 1973; Leach and Taylor 1982; Foster and Moon 1986) because it reduces the stress associated with regular netting and air exposure. Recently, palm oil implants have been used to administer cortisol (Vijayan and Leatherland 1989). This method is an improvement over daily IP injections, but it does not permit the precise determination of the rate at which the hormone is continually delivered. By knowing the metabolic clearance rate of cortisol in trout (e.g. Brown et al. 1986), it is possible to fill the pumps with an appropriate concentration of cortisol to maintain the desired plasma cortisol concentration (Fig. 1 and 2). The target plasma cortisol concentrations used in this study were 100 and 200 ng·mL^{-1}. These values are reasonable estimates of the physiological cortisol status one might expect in a fish subjected to a substantial chronic stress (e.g. Brown et al. 1984).

The present study is the first to monitor cortisol levels on a daily basis throughout the period of cortisol administration. To date, no other study has ensured that cortisol is at a steady state for the entire experimental period. Foster and Moon (1986) clearly demonstrated that, rather than maintaining a steady

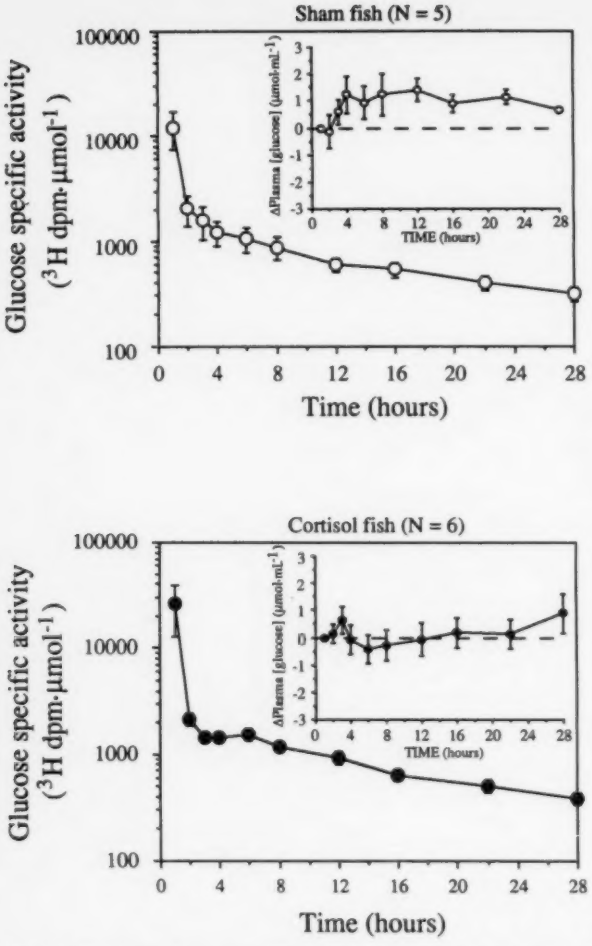

FIG. 3. Change in glucose specific activity in sham and cortisol-treated (administered at 4.2 $\mu g \cdot h^{-1} \cdot 100\ g^{-1}$) fed rainbow trout. Inset represents means of normalized plasma glucose concentrations. Vertical bars through each point represent 1 SEM; error bars in cortisol fish are within size of symbol.

state, daily IP cortisol injections resulted in massive increases in plasma cortisol levels well above controls to values significantly below controls between injections. Yet, it has been shown that neither acute stress (Goss and Wood 1988) nor chronic stress (Brown et al. 1984) results in such massive oscillations in plasma cortisol levels.

There is clearly a postsurgery surge (12–72 h) in both plasma cortisol and glucose, especially in fed trout (Figs. 1 and 2). The cortisol surge is due to endogenous cortisol release because both cortisol and sham fish respond similarly. Furthermore, the mini-osmotic pumps only attained their desired pumping rate after approximately 24 h following implantation (based upon information supplied by Alza Corp.). The accompanying hyperglycaemia presumably results from the stress associated with the surgical procedure (possibly related to catecholamine release) and subsides within 72 h pump insertion.

The effect of cortisol administration on plasma metabolites other than glucose (Tables 1 and 3) was consistent with the data previously reported in the literature (Chan and Woo 1978; Lidman et al. 1979; Leach and Taylor 1982; Vijayan and Leatherland 1989), although Lidman et al. (1979) reported a significant effect of cortisol on plasma lactate. In mammals, it is well documented that cortisol stimulates the catabolism of skeletal muscle, resulting in an elevation of circulating plasma amino acids (Leung and Munck 1975; Kraus-Friedmann 1984). The finding that circulating amino acids were elevated by cortisol above sham levels by 216 h in the present study was, therefore, predicted. Chan and Woo (1978) also reported that plasma amino acids were elevated by cortisol treatment whereas Leach and Taylor (1982) found that in *F. heteroclitus*, circulating amino acids were unaffected by elevated cortisol. The increased plasma amino acids may increase gluconeogenesis by providing additional substrates. This study cannot determine the precise impact of this effect.

Previous studies have reported that cortisol administration elicits a hyperglycaemic response in teleost fishes. The precise source of this increased plasma glucose is unclear. A decreased glucose utilization was first reported by McDonald and Than (1976) in a marsupial during glucocorticoid treatment. Cortisol treatment in dogs, however, did not affect the rate of glucose turnover (Hetenyi et al. 1983). Leach and Taylor (1982) found no breakdown of muscle protein or liver glycogen, suggesting that decreased glucose utilization in peripheral tissue accounted for the hyperglycaemia in killifish. More recently, Vijayan and Leatherland (1989) expressed support for a decreased glucose utilization in their study on coho salmon *Oncorhynchus kisutch*, even though glycogen levels declined significantly. The findings of that study are particularly interesting when compared with our study, since daily IP injections were avoided by using coconut oil implants containing cortisol. The reason for these contrasting results may simply reflect interspecific differences or differences in the temporal resolution of the two studies (salmon versus trout). In addition, Vijayan and Leatherland (1989) suggested that a threshold level of cortisol must be attained for gluconeogenic stimulation, and perhaps our fish never reached this threshold. We provide evidence in trout that the glucose turnover is unaffected by chronically elevated cortisol treatment (Fig. 3), eliminating this as a possible explanation for the absence of a glycaemic response in this study.

Another possibility is that hyperglycaemia in fish is due to elevated plasma cortisol enhancing liver gluconeogenesis, yet among previous studies (e.g. Chan and Woo 1978; Lidman et al. 1979; Leach and Taylor 1982; Vijayan and Leatherland 1989), only that by Foster and Moon (1986) has directly examined the role of cortisol on liver metabolism. Foster and Moon (1986) found that the actual flux of gluconeogenic precursors (lactate, alanine, and aspartate) to glucose in hepatocytes isolated from cortisol-injected American eels was decreased compared with control. Consistent with a decrease in hepatic gluconeogenesis was a hypoglycaemia in the cortisol-treated eels. Butler (1968) showed that in hypophysectomized American eels, 10 d of cortisol treatment did not induce a hyperglycaemic response, but if the treatment was continued for 21 d, hyperglycaemia developed. Clearly, the presumed role of a purported stress hormone must be questioned if 21 d is required to elicit a response. Our finding that initial hepatocyte glycogen levels were lower in cortisol-treated fish than in shams is difficult to explain, since glycogen phosphorylase activity and the activities of other key regulatory enzymes involved in the intermediate carbohydrate metabolism (Table 2) were unchanged by elevated cortisol. Likewise, cortisol treatment had no effect on the flux of the gluconeogenic precursors, lactate and alanine,

to glucose or any other metabolite estimated (Table 4), supporting the study in eels by Foster and Moon (1986). Consistent with this finding was the fact that the levels of plasma glucose (Fig. 1 and 2) were not related to those of cortisol. So even though amino acids in cortisol-treated trout were significantly above those of controls, this did not result in hyperglycaemia.

These experiments provide no evidence that cortisol possesses glucocorticoid activity in rainbow trout. To arrive at this conclusion, we have not only examined the glycaemic status of cortisol-treated fish, but have also investigated the activities of key metabolic enzymes, the flux of gluconeogenic precursors, and the rate of glucose turnover. Of particular interest is the finding that cortisol elevates plasma amino acids. This implies that cortisol, alone, can increase the supply of usable gluconeogenic substrates by stimulating peripheral muscle catabolism, but the hormone does not alter the manner in which amino acids are then metabolized. Since physiological levels of cortisol, mimicking the corticoid status of stressed rainbow trout, were maintained throughout these experiments, it must be concluded that some other stress-related factor, either alone or in conjunction with cortisol, causes the hyperglycaemia in stressed fish. Every attempt was made in this study to elevate cortisol in otherwise unstressed fish. This was considered necessary to avoid any permissive effects that cortisol may have on other stress hormones. It is possible that the disturbance associated with daily injections is sufficiently stressful to elicit a generalized stress response, which then interacts with cortisol to produce hyperglycaemia. Further research is needed to better understand the metabolic effects resulting uniquely from cortisol and those which result from permissive effects of cortisol on other stress hormones.

Acknowledgements

These studies were made possible by operating grants from the Ontario Ministry of Natural Resources, World Wildlife Toxicology Fund, and Natural Sciences and Engineering Research Council of Canada to T.W.M. and S.F.P. D.E.A. was supported in part by the above grants and a FCAR Fellowship. The authors gratefully acknowledge Glen D. Foster for his help during the experiments and for suggestions during the preparation of this manuscript.

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CJFSDX48(9) 1605–1817 (1991)
ISSN 0706-652X

Subscription rates/Abonnements

Canada: Institutional/Collectif $210; Personal/Personnel $85
Other countries/Autres pays : Institutional/Collectif $252(U.S.); Personal/Personnel $102(U.S.)
Single issue/Par numéro : $14.95 (Canada); $17.95(U.S.) (Other countries/Autres pays)